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Synthesis of new chemical entities from paracetamol and NSAIDs with improved pharmacodynamic profile

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Abstract—It was envisaged to combine high antipyretic activity of paracetamol into commonly used NSAIDs. To achieve this goal new chemical entities were synthesized by chemically combining paracetamol and NSAIDs, and biologically evaluated for their antipyretic, analgesic, anti-inflammatory and ulcerogenic potential. The acid chloride of parent NSAIDs was reacted with excess of *p*-aminophenol to yield the desired *p*-amidophenol derivatives (**1B–7B**). Acetate derivatives (**1C–7C**) of these phenols (**1B–7B**) were also prepared by their treatment with acetic anhydride, in order to see the impact of blocking the free phenolic group on the biological activity of the derivatives. All the synthesized *p*-amidophenol derivatives showed improved antipyretic activity than paracetamol with retention of anti-inflammatory activity of their parent NSAIDs. These compounds elicited no ulcerogenicity unlike their parent drugs.

1. Introduction

Paracetamol is a commonly used analgesic and antipyretic drug. However, it has only weak anti-inflammatory effects. The failure of paracetamol to exert anti-inflammatory activity may be attributed to the fact that paracetamol is only a weak inhibitor of COX in the presence of high concentration of the peroxides that are found in the inflammatory lesions.[1] In contrast, its antipyretic effect may be explained by its ability to inhibit COX in the brain, where peroxide tone is low.[2] Single or repeated therapeutic doses of paracetamol have no effect on the cardiovascular and respiratory system nor does the drug produce gastric irritation/erosion. Recent reports[3,4] suggest that overdose of paracetamol is the most common cause of acute liver failure (ALF) in United States and United Kingdom.

Nonsteroidal anti-inflammatory agents (NSAIDs) are the most commonly prescribed drugs in the world but, their use as anti-inflammatory, antipyretic, antithrombotic and analgesic agents continues to be limited due to their undesired side effects mainly on the gastrointestinal (GI) tract. NSAIDs are known to have inhibitory activity for both the isoforms of the cyclooxygenase (COX) enzyme. They vary considerably in their tendency to cause gastric erosions and ulcers.[1] Gastric damage by these agents is caused by at least two distinct mechanisms. One is by inhibiting the cytoprotective COX-1 in the stomach and second by physical contact and ion-trapping mechanism.[5,6] The use of prodrugs to temporarily mask acidic group of NSAIDs has been postulated as an approach to decrease the GI toxicity due to direct contact effect.[7,8] These prodrugs release the parent moieties after absorption by undergoing enzymatic/chemical hydrolysis. On the other hand selective inhibition of the inducible COX-2 enzyme, sparing the constitutive COX-1, formed the basis for designing of COXIBs with minimum degree of ulcerogenic risk. This new concept of treating inflammation related disease came into effect with the consecutive launch of celecoxib[9] and rofecoxib.[10,11]

Willoughby and colleagues[12] described the effect of some selective COX-2 inhibitors and some dual (COX-1 and COX-2) inhibitors on carrageenan pleurisy in the rat over a time period ranging from 0 to 48 h after injection of the irritant. This investigation gave surprising results. The COX-2 inhibitors showed anti-inflammatory activity early in the inflammatory response, coincident with the expression

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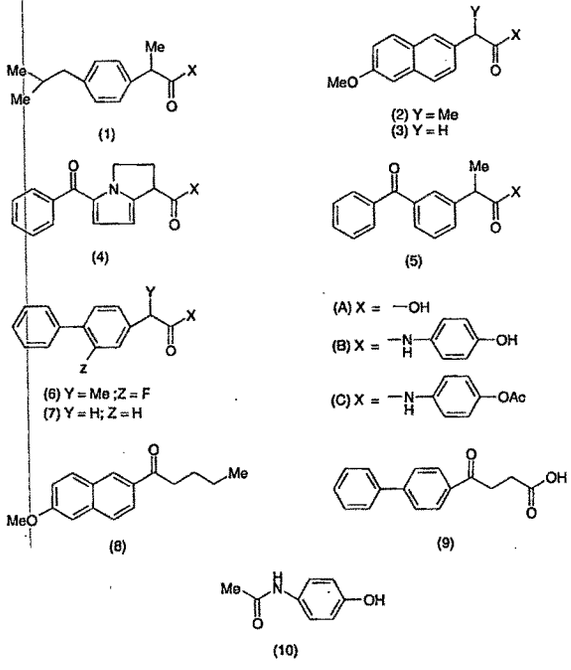
of COX-2 protein as did the older dual inhibitors such as indomethacin. However, by 6 h the COX-2 inhibitors were without effect although the dual inhibitors still showed efficacy. At this point the COX-2 protein as shown by Western blotting was no longer present. This study showed the supremacy of conventional NSAIDs over the COXIBs for chronic use. Moreover, withdrawal of rofecoxib from market recently by its originators due to adverse cardiovascular effects puts a big question mark on the safety profile of other COXIBs in the long-term therapy.

It has been reported[13] that conversion of the carboxylic group containing NSAIDs to ester and amide functions makes them more selective towards COX-2 enzyme. Taking the above said report and the potential cardiovascular dangers posed by COXIBs into cognizance, and the cost and time involved in the discovery of a new drug, it was thought of converting some common NSAIDs into *p*-amidophenol derivatives. *p*-Aminophenol has been evaluated in the past[1] as analgesic–antipyretic but its N-acetylated derivative, that is, paracetamol (**10**) has been found to be the most suitable, therapeutically. It was planned to substitute the acetyl group in paracetamol (**10**) with the carboxylic group containing NSAIDs as the acyl groups. Threefold advantages were visualized through such chemical conversion. First, the free carboxylic group present in the NSAIDs would be blocked by a nonhydrolysable (at physiological pH) amide linkage thereby preventing the local contact mechanism, which was partially responsible for GIT ulceration by these NSAIDs. Second, these new derivatives are expected to show more selectivity towards COX-2 enzyme as reported[13] for the amide derivatives for NSAIDs, resulting in further reduction in ulcerogenicity of the parent NSAIDs. Finally, due to the structural resemblance of these amides with paracetamol they are expected to exhibit substantial antipyretic effect like the parent drug paracetamol (**10**).



2. Results and discussion

2.1. Chemistry

Commonly used carboxylic group containing NSAIDs like ibuprofen (**1A**) naproxen (**2A**), 6-methoxy-2-naphthylacetic acid (6-MNA) (**3A**) (active metabolite of nabumetone (**8**)), ketorolac (**4A**), ketoprofen (**5A**), flurbiprofen (**6A**) and biphenylacetic acid (**7A**) (active metabolite of fenbufen (**9**)) were chosen for this study. These compounds were converted into the acid chlorides by treatment with thionyl chloride under anhydrous conditions. The acid chloride was reacted with excess of *p*-aminophenol in dioxane to yield a mixture containing the desired *p*-amidophenol derivatives (**1B–7B**) and the undesired 4-aminophenyl esters. The unwanted 4-aminophenyl esters were removed by washing the chloroform solution of the mixture continuously with dilute hydrochloric acid until it became free from it. The products so obtained were purified by repeated recrystallizations. All the synthesized *p*-amidophenol derivatives were converted into their acetates in order to see the impact of blocking the free phenolic group on the biological activity of the derivatives. The acetate derivatives (**1C–7C**) were prepared by treatment of the free phenolic compounds (**1B–7B**) with acetic anhydride in pyridine. The synthesized compounds conform to the assigned structures, as deduced on the basis of their spectral and elemental data. Biphenylacetic acid (**7A**) was prepared by Friedel–Crafts acetylation of biphenyl followed by Kindler-modified Willgerodt reaction of the 4-acetyl derivative.[14] Similarly, 6-MNA (**3A**) was obtained by carrying out Friedel–Crafts acetylation of nerolin[15] and then submitting the product so obtained to Kindler-modified Willgerodt reaction to obtain the desired product (**3A**).[16]

2.2. Biological

The compounds (**1B–7C**) were synthesized with a view to incorporate the antipyretic activity component of paracetamol (**10**) into the NSAIDs with their normal anti-inflammatory activity but without GIT ulceration. So, all the synthesized derivatives (**1B–7C**) were evaluated for their antipyretic activity in animal model[17] using lipopolysaccharide (LPS) (from *Escherichia coli*) endotoxin for producing pyrexia. Percent reversal of the body temperature (antipyretic activity) was determined (Table 1) using paracetamol (**10**) as the standard antipyretic drug. Interesting results were obtained as shown in Figure 1. All the compounds with free phenolic group have shown superior antipyretic activity than paracetamol (**10**). It may be noted that this activity was determined on equal weight bases with paracetamol for all the compounds. That means the *p*-amidophenol component in the derivatives (**1B–7C**) on molar basis is much less than that present in paracetamol (**10**). But, free phenolic group seems to be essential in these compounds also, like paracetamol (**10**) for superior antipyretic activity as the acylation of the phenolic hydroxyl group has led to decrease in antipyretic activity.

COX-2 inhibiting activity for the synthesized compounds was determined using the Cayman COX-2

Table 1. Biological activities of synthesized compounds

Compound	% inhibition of COX-2 at 22 μM concn	Anti-inflammatory activity		Ulcerogenic potential		Analgesic activity		Antipyretic activity	
		Dose mg/kg	% inhibition	Dose mg/kg	Ulcer index	Dose mg/kg	% inhibition	Dose mg/kg	Pyrexia % reversal of body temperature
1A	8.4	20	60.0	200	0.5013 ± 0.021	20	41.9	—	ND
1B	10.4	28.8	34.8	290	Nil	28.8	19.1	25	87.4
								10	49.3
1C	NI	32.9	38.7	330	Nil	32.9	36.5	25	75.0
								10	43.5
2A	81.8	20	81.1	200	0.981 ± 0.038	20	43.0	—	ND
2B	12.7	27.9	39.4	280	Nil	27.9	20.7	25	59.5
								10	25.7
2C	NI	31.6	27.6	316	Nil	31.6	21.8	25	12.5
3A	10.5	20	55.4	200	0.630 ± 0.052	20	42.2	—	ND
3B	8.9	28.4	44.6	284	Nil	28.4	30.8	25	75.0
3C	NI	32.3	41.5	323	Nil	32.3	28.5	25	68.7
4A	100	10	75.0	75	0.484 ± 0.030	20	54.1	—	ND
4B	31.8	13.5	86.4	101.3	Nil	25	52.6	25	55.8
4C	11.1	15.2	83.5	114	Nil	30.4	58.2	25	37.5
5A	100	20	86.7	200	0.822 ± 0.039	25	74.8		ND
5C	4.3	29.5	72.3	295	Nil	36.9	68.4	25	50.0
6A	15.4	8	84.0	25	0.80 ± 0.048	10	54.9	—	ND
6B	12.3	11	68.9	34.4	Nil	13.1	45.8	25	58.5
6C	NI	12.4	58.1	38.8	Nil	15.5	52.3	25	18.5
7A	10.5	10	60.9	500	0.564 ± 0.069	25	51.7	—	ND
7B	8.9	14.3	57.9	715	Nil	35.8	47.8	25	82.4
7C	NI	16.3	59.6	815	Nil	40.8	45.2	25	67.5
								10	35.5
10	—	—	—	—	—	—	—	25	63.8
								10	39.9

NI, no inhibition at the test dose; ND, not determined.

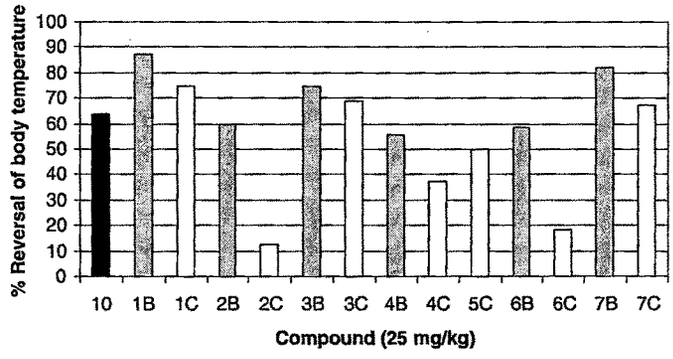


Figure 1. Antipyretic activity of synthesized compounds in rats.

colorimetric screening kit.[18] No uniform conclusion could be drawn on the basis of this study except that all the acetylated derivatives showed no inhibition at the test dose level. So, it was planned to perform anti-inflammatory activity in the in vivo model using carrageenan-induced paw edema.[19] The activity was carried out on equimolar basis to the parent drug. The results of the study (Table 1) indicate that the conversion of free carboxylic group in the NSAIDs to the amide linkage caused reduction in the anti-inflammatory activity of the parent drugs, in general. However, ketorolac (derivative **4B**) was found to be an exception where an increase in inflammatory activity was noticed. Acetylation of free phenolic group was observed not to change this activity drastically. Since NSAIDs do possess peripheral analgesic activity, the synthesized derivatives were evaluated for this activity using the writhing method[20] in mice. No straightforward conclusion could be drawn from this study except that all the synthesized derivatives possessed analgesic activity with somewhat less potency than their parent NSAIDs.

Ulcerogenic potential of the synthesized compounds was determined in rat model.[21] It was very encouraging to note that none of the compounds showed any ulcer formation in the test animals. This may be due to the dual factors of blocking of the free acidic group and more selectivity of these compounds to inhibit COX-2.

3. Conclusion

Conversion of the conventional carboxylic group containing NSAIDs into *p*-amidophenol derivatives has resulted into new potential drugs having much improved antipyretic activity. These compounds also possessed sufficiently good potency to be used as anti-inflammatory drugs with nil ulcerogenicity in acute model of this biological testing. Keeping in view the therapeutic superiority of the classical NSAIDs over COXIBs on longer duration of usage, newer potential compounds have been developed possessing high analgesic–antipyretic activity with nil/minimum GIT ulceration. Acetylation of free phenolic group in these synthesized compounds was not found to be a suitable proposition as it decreased the potency.

4. Experimental

4.1. Chemistry

Melting points were determined using a heating block-type melting point apparatus and are uncorrected. Purity of the compounds was ascertained by thin-layer chromatography (TLC) on silica gel plates (60 F_{254}; Merck), visualizing with ultraviolet light or iodine vapors. The yields reported here are unoptimized. IR spectra were recorded using KBr disc method on a Shimadzu FT-IR Model 8300. ^{1}H NMR spectra on a Brucker 300 MHz spectrometer were recorded in $CDCl_3$ (chemical shifts in δ ppm). Assignment of exchangeable protons (N*H*) was confirmed by D_2O exchange studies. Elemental analyses were obtained on Carlo Erba, Italy, and Perkin-Elmer instruments. All the compounds were purified by recrystallization from acetone–pet. ether.

4.2. General procedure for the preparation of compounds 1B–7B

4.2.1. Representative preparation of 4-[2-(4-isobutylphenyl)propionamido]phenol (1B). Ibuprofen **1A** (2 g) was dissolved in dry toluene (25 ml) and thionyl chloride (2 ml) was added dropwise with constant stirring. The reaction mixture was heated at 80 °C on a water bath for 2 h, and excess thionyl chloride and toluene was removed under vacuum. The residue so obtained was dissolved in dry dioxane (25 ml) and added dropwise to a solution of *p*-aminophenol (5 g) in dioxane (50 ml) with stirring. The reaction mixture was stirred for 1 h at room temperature and heated on water bath for 3 h. Excess of dioxane was removed under vacuum, the reaction mixture acidified with dilute hydrochloric acid and extracted with chloroform (3× 50 ml). The combined organic extract was washed successively with hydrochloric acid (5%) until free from the basic impurities, dried over sodium sulfate and solvent removed. The residue so obtained was purified by crystallization from acetone–pet. ether. Yield 50%; mp 112–115 °C; IR (KBr): 3299, 1653, 1536, 1510, 1234, 827 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.90 (d, 6H), 1.59 (d, 3H), 1.85 (m, 1H), 2.45 (d, 2H), 3.68–3.71 (q, 1H), 6.95 (br s, 1H), 6.68–6.73 (m, 2H), 7.10–7.15 (m, 4H), 7.28–7.31 (m, 2H). Anal. Calcd for $C_{19}H_{23}NO_2$: C, 76.74; H, 7.80; N, 4.71. Found: C, 76.48; H, 7.53; N, 4.38.

4.2.2. 4-[2-(6-Methoxy-2-naphthyl)propionamido]phenol (2B). Yield 35%; mp 156–159 °C; IR (KBr): 3327, 1652, 1539, 1512, 1226, 826 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.60 (d, 3H), 3.86–3.89 (q, 1H), 3.91 (s, 3H), 8.90 (br s, 1H), 6.65–6.69 (m, 2H), 7.10–7.15 (m, 1H), 7.32–7.55 (m, 4H), 7.69–7.77 (m, 3H). Anal. Calcd for $C_{20}H_{19}NO_3$: C, 74.75; H, 5.96; N, 4.36. Found: C, 74.94; H, 6.19; N, 4.52.

4.2.3. 4-[2-(6-Methoxy-2-naphthyl)acetamido]phenol (3B). Yield 38%; mp 201–203 °C; IR (KBr): 3285, 1657, 1539, 1512, 825 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 3.68 (s, 2H), 3.85 (s, 3H), 6.64–6.67 (m, 2H), 7.10–7.14 (m, 1H), 7.33–7.43 (m, 4H), 7.70–7.77 (m, 3H), 9.15 (br s, 1H), 9.92 (br s, 1H). Anal. Calcd for $C_{19}H_{17}NO_3$: C, 74.25; H, 5.58; N, 4.56. Found: C, 73.91; H, 5.24; N, 4.28.

4.2.4. 5-Benzoyl-*N*-(4-hydroxyphenyl)-2,3-dihydro-1*H*-pyrrolizine-1-carboxamide (4B). Yield 43%; mp 228–229 °C; IR (KBr): 3300, 1661, 1651, 1513, 1240, 725 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.81 (m, 2H), 4.07 (m, 1H), 4.52 (m, 2H), 6.09 (br s, 1H), 6.64–6.68 (m, 2H), 6.89–6.90 (d, 1H), 7.44–7.57 (m, 6H), 7.81–7.85 (m, 2H). Anal. Calcd for $C_{21}H_{18}N_2O_3$: C, 72.82; H, 5.24; N, 8.09. Found: C, 72.99; H, 5.52; N, 8.27.

4.2.5. 4-[2-(3-Benzoylphenyl)propionamido]phenol (5B). Sticky material. Yield 47%; IR (KBr): 3286, 1652, 1535, 1510, 834 cm^{-1}.

4.2.6. 4-[2-(2-Fluoro-4-biphenyl)propionamido]phenol (6B). Yield 40%; mp 170–173 °C; IR (KBr): 3291, 1652, 1539, 1531, 1237, 827 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.61 (d, 3H), 3.68–3.71 (q, 1H), 6.97 (br s, 1H), 6.74–6.77 (m, 2H), 7.16–7.23 (m, 2H), 7.28–7.47 (m, 6H), 7.53–7.55 (m, 2H). Anal. Calcd for $C_{21}H_{18}FNO_2$: C, 75.21; H, 5.41; N, 4.18. Found: C, 75.66; H, 5.22; N, 4.37.

4.2.7. 4-[2-(4-Biphenyl)acetamido]phenol (7B). Yield 15%; mp 205–208 °C; IR (KBr): 3246, 1651, 1546, 1514, 1245, 749 cm^{-1}; ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 3.68 (s, 2H), 7.30 (br s, 1H), 6.72–6.75 (m, 2H), 732–7.45 (m, 7H), 7.53–7.58 (m, 4H), 9.24 (br s, 1H). Anal. Calcd for $C_{20}H_{17}NO_2$: C, 79.19; H, 5.65; N, 4.62. Found: C, 79.49; H, 5.83; N, 4.38.

4.3. General procedure for the preparation of compounds 1C–7C

4.3.1. Representative preparation of 4-[2-(4-isobutylphenyl)propionamido]phenyl acetate (1C). Compound **1B** (0.4 g) was dissolved in dry pyridine (1 ml) and cooled in ice bath. Acetic anhydride (1.0 ml) was added dropwise with stirring. Reaction mixture was heated on water bath for 3 h and allowed to cool to room temperature. It was poured over crushed ice containing conc hydrochloric acid (3 ml). The solid was filtered, dried and recrystallized from acetone–pet. ether. Yield 85%; mp 145–147 °C; IR (KBr): 3354, 1734, 1684, 1541, 1510, 839 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.90 (d, 6H), 1.59 (d, 3H), 1.85 (m, 1H), 2.26 (s, 3H), 2.46 (d, 2H),

3.67–3.70 (q, 1H), 7.21 (br s, 1H), 6.96–7.01 (m, 2H), 7.10–7.16 (m, 4H), 7.39–7.44 (m, 2H). Anal. Calcd for $C_{21}H_{25}NO_3$: C, 74.31; H, 7.42; N, 4.13. Found: C, 74.68; H, 7.21; N, 4.01.

4.3.2. 4-[2-(6-Methoxy-2-naphthyl)propionamido]phenyl acetate (2C). Yield 90%; mp 212–216 °C; IR (KBr): 3380, 1748, 1668, 1528, 1502, 857 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.60 (d, 3H), 2.25 (s, 3H), 3.86–3.89 (q, 1H), 3.91 (s, 3H), 8.97 (br s, 1H), 6.95–7.00 (m, 2H), 7.09–7.14 (m, 1H), 7.38–7.54 (m, 4H), 7.66–7.78 (m, 3H). Anal. Calcd for $C_{22}H_{21}NO_4$: C, 72.71; H, 5.82; N, 3.85. Found: C, 72.52; H, 5.62; N, 3.67.

4.3.3. 4-[2-(6-Methoxy-2-naphthyl)acetamido]phenyl acetate (3C). Yield 84%; mp 175–176 °C; IR (KBr): 3305, 1750, 1651, 1539, 1506, 839 cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.27 (s, 3H), 3.67 (s, 2H), 3.85 (s, 3H), 6.99–7.03 (m, 2H), 7.11–7.15 (m, 1H), 7.33–7.47 (m, 4H), 7.70–7.77 (m, 3H) 9.06 (br s, 1H). Anal. Calcd for $C_{21}H_{19}NO_4$: C, 72.19; H, 5.48; N, 4.01. Found: C, 72.49; H, 5.64; N, 3.88.

4.3.4. *N*-(4-Acetoxyphenyl)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxamide (4C). Yield 89%; mp 197–198 °C; IR (KBr): 3260, 1755, 1661, 1539, 1508, 720 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.28 (s, 3H), 2.80 (m, 2H), 4.08 (m, 1H), 4.53 (m, 2H), 6.17 (br s, 1H), 6.89–6.90 (d, 1H), 7.04–7.06 (m, 2H), 7.45–7.57 (m, 6H), 7.82–7.85 (m, 2H). Anal. Calcd for $C_{23}H_{20}N_2O_4$: C, 71.12; H, 5.19; N, 7.21. Found: C, 71.48; H, 5.41; N, 7.52.

4.3.5. 4-[2-(3-Benzoylphenyl)propionamido]phenyl acetate (5C). Yield 49%; mp 87–88 °C; IR (KBr): 3357, 1730, 1683, 1661, 1538, 1508, 838 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.62 (d, 3H), 2.27 (s, 3H), 3.72–3.76 (q, 1H), 7.18 (br s, 1H), 6.98–7.02 (m, 2H), 7.44–7.52 (m, 5H), 7.57–7.65 (m, 2H), 7.68–7.72 (m, 1H), 7.78–7.82 (m, 3H). Anal. Calcd for $C_{23}H_{21}NO_4$: C, 73.58; H, 5.64; N, 3.73. Found: C, 73.79; H, 5.38; N, 3.49.

4.3.6. 4-[2-(2-Fluoro-4-biphenyl)propionamido]phenyl acetate (6C). Yield 90%; mp 165–168 °C; IR (KBr): 3357, 1732, 1684, 1539, 1509, 764 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.62 (d, 3H), 2.27 (s, 3H), 3.68–3.71 (q, 1H), 7.09 (br s, 1H), 6.98–7.03 (m, 2H), 7.16–7.23 (m, 2H), 7.32–7.49 (m, 6H), 7.52–7.56 (m, 2H). Anal. Calcd for $C_{23}H_{20}FNO_3$: C, 73.20; H, 5.34; N, 3.71. Found: C, 72.77; H, 5.06; N, 3.95.

4.3.7. 4-[2-(4-Biphenyl)acetamido]phenyl acetate (7C). Yield 90%; mp 185–188 °C; IR (KBr): 3176, 1751, 1646, 1549, 1501, 746 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.26 (s, 3H), 3.77 (s, 2H), 7.15 (br s, 1H), 6.98–7.03 (m, 2H), 733–7.48 (m, 7H), 7.57–7.63 (m, 4H). Anal. Calcd for $C_{22}H_{19}NO_3$: C, 76.50; H, 5.54; N, 4.06. Found: C, 76.84; H, 5.81; N, 3.72.

4.4. Antipyretic activity

Antipyretic activity of synthesized compounds (**1B–7C**) was determined on rats using lipopolysaccharide (LPS) (from *E. coli*) endotoxin for producing pyrexia in rat.[17] Male Sprague–Dawley rats (150–200 g) were fasted for 16–18 h before use and were divided into parallel groups ($n = 5$). The animals were placed temporarily in restrainer and the resting rectal temperature was recorded using a flexible temperature probe connected to Bio-Pac data acquisition system. The same probe and system were used for all animals to reduce experimental error. The animals were returned to their respective cages after the temperature measurement. Animals were injected either normal saline or LPS (0.36 mg/kg, Sigma, USA) intraperitoneally and the rectal temperature was measured at 0, 5, 6 and 7 h after LPS injection. At 5 h when the increase in rectal temperature had reached a plateau, the LPS injected rats were given orally either vehicle (1% CMC) or test compound (suspended in 1% CMC) to determine whether the rise in temperature could be reversed. Percent reversal (Antipyretic activity) was calculated using the rectal temperature obtained at 7 h taking this value in the vehicle control group as zero reversal.

4.5. In vitro COX inhibition assay

COX-2 inhibiting activity for the synthesized compound and parent drugs was determined[18] using the colorimetric ovine cyclooxygenase (COX) assay kit (Cayman Chemical Company, MI, USA). This assay analyzes the peroxidase activity of the enzymes using *N,N,N′,N′*-tetramethylphenylenediamine (TMPD) as the reducing co-substrate.[22] Valdecoxib was used as the positive control for COX-2 inhibition. The compounds were dissolved in DMSO and diluted in the assay buffer before use. The assays were run according to the manufacturer's instructions.

4.6. In vivo carrageenan-induced rat paw edema assay

Anti-inflammatory activity was determined by using carrageenan-induced rat paw edema method described by Winter et al.[19] Fasted male Sprague–Dawley rats (150–200 g) were divided in parallel groups ($n = 5$) and were given orally either the vehicle (1% CMC) or test compound as suspension in 1% CMC. The activity was carried out on equimolar basis to the parent drug. A line was drawn using permanent marker at the ankle of the left hind paw to define the arc of the paw to be monitored. The paw volume (V_{0h}) was measured using a Plethysmometer (Ugo-Basile, Italy). The animals were then injected subplantarly with 50 µl of 1% carrageenan (Sigma, USA) in normal saline solution (i.e., 500 µg carrageenan per paw). Three hours after the carrageenan injection, the paw volume (V_{3h}) was measured, and the increase in paw volume ($V_{3h} - V_{0h}$) was calculated. The increase in paw volume was compared with that in the vehicle control group, and percent inhibition was calculated taking the values in the control group as 0% inhibition.

4.7. Analgesic activity

Analgesic activity of the derivatives was evaluated using the acetic acid writhing model in mice as described by Koster et al.[20] Swiss albino mice (18–25) divided in

parallel groups ($n = 6$) were given orally either the vehicle (1% CMC) or test compound as suspension in 1% CMC. After 1 h of oral administration the writhing syndrome was elicited by intraperitoneal administration of acetic acid (10 ml/kg body weight, 0.7% in normal saline) and number of writhes for each mice was counted after 5 min of injection for a period of 20 min. The average number of writhes in each group of compound treated mice was compared with that of the control and degree of analgesia was expressed as percent inhibition calculated according to the formula:

$$\% \text{ inhibition of writhing} = (1 - T/S) \times 100,$$

where S is the number of writhes in control group of mice and T is the number of writhes in compound treated group of mice.

4.8. Ulcerogenic effect[21]

Sprague–Dawley rats ($n = 6$) of either sex were fasted for 36 h with water ad libitum prior to administration of the derivative. The animals were further kept on fasting for 4 h after dosing. The derivatives as suspension in 1% CMC or vehicle (1% CMC) were administered orally. The animals were sacrificed by cervical dislocation and their stomach was dissected out, cut along the greater curvature, washed with normal saline and kept in formalin solution (5%) for 15 min and gastric mucosa was observed for the lesions using a 2×2 binocular magnifier and the ulcer index was determined using the following formula as given below:

$$\text{Ulcer index} = 10(\text{Au}/\text{Am}),$$

where $\text{Au} = A_l + \text{Ac} + \text{Ap}$, A_l is the area of linear lesions ($l * b$), Ac is the area of circular lesions (Πr^2), Ap is the total no. of petechiae/5, and Am is the total mucosal area ($\Pi D^2/8$) (D = diameter of stomach).

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Original research paper

Studies in 3,4-diaryl-1,2,5-oxadiazoles and their *N*-oxides: Search for better COX-2 inhibitors

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A series of 3,4-diaryl-1,2,5-oxadiazoles and 3,4-diaryl--1,2,5-oxadiazole *N*-oxides were prepared and evaluated for COX-2 and COX-1 binding affinity *in vitro* and for anti--inflammatory activity by the rat paw edema method. *p*--Methoxy (*p*-OMe) substituted compounds **9**, **21**, **34**, **41**, **42** showed COX-2 enzyme inhibition higher than that showed by compounds with other substituents. 3,4-Di(4--methoxyphenyl)-1,2,5-oxadiazole *N*-oxide (**42**) showed COX-2 enzyme inhibition of 54% at 22 μmol L^{-1} and COX-1 enzyme inhibition of 44% at 88 μmol L^{-1} concentrations, but showed very low *in vivo* anti-inflammatory activity. Its deoxygenated derivative (**21**) showed lower COX-2 enzyme inhibition (26% at 22 μmol L^{-1}) and higher COX-1 enzyme inhibition (53% at 88 μmol L^{-1}) but, marked *in vivo* anti-inflammatory activity (71% at 25 mg kg^{-1}) *vs.* celecoxib (48% at 12.5 mg kg^{-1}). Molecular modeling (docking) studies showed that the methoxy group is positioned in the vicinity of COX-2 secondary pocket and it also participates in hydrogen bonding interactions in the COX-2 active site. These preliminary studies suggest that *p*-methoxy (*p*-OMe) group in one of benzene rings may give potentially active leads in this series of oxadiazole/*N*-oxides.

Keywords: 1,2,5-oxadiazole, 1,2,5-oxadiazole *N*-oxide, COX-2 inhibitor

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Discovery of two cyclooxygenase (COX) isoenzymes, a constitutive COX-1, serving as homeostatic prostanoid producing agent, and COX-2, responsible for pro-inflammatory prostanoid production, led to the development of new nonsteroidal anti-inflammatory drugs (NSAIDs), selective COX-2 inhibitors, promising minimal NSAID-typical toxicity with full anti-inflammatory efficacy. COX-2 inhibitors have been successful in treating inflammatory diseases like acute pain, rheumatoid arthritis and osteoarthritis; a few of them are also being studied for treating different types of cancer and Alzheimer's disease (1). Despite a few recent cautionary reports, the coxib treatment has a high degree of benefit over risk, and strategies for using NSAIDs have been described by Antman *et al.* (2).

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A wide variety of carbocycles and heterocycles can serve as templates for COX-2 inhibitors, *i.e.*, cyclopentene [SC-57666] (3) pyrazole [celecoxib (4), SC-58125 (5)], furan [rofecoxib (6)], isoxazole [valdecoxib (7), paracoxib sodium (8)], and pyridine [etoricoxib (9)]. In an ongoing research program in this department on COX-2 inhibitors with improved biological profile, we synthesized (10) a series of 3,4-diaryl-1,2,5-oxadiazoles and 3,4-diaryl-1,2,5-oxadiazole *N*-oxides. The compounds are novel in that the vicinal diaryl heterocyclic (five membered ring) pharmacophore of the coxibs has been incorporated with the nitric oxide releasing group (1,2,5-oxadiazole *N*-oxide) into one single entity in the compounds synthesized. In this paper, we report the synthesis, preliminary biological evaluation and molecular docking studies of some of these oxadiazoles and their *N*-oxides as selective COX-2 inhibitors.

1,2,5-oxadiazole *N*-oxides (furoxans) are reported (11) to be thiol dependent NO donors, whose biological activity is produced by action on the soluble guanylate cyclase--cyclic guanosine monophosphate (sGC-cGMP) pathway. Furoxans are considered to possess favorable bioactivity since they cause a slow release of NO resulting in longer duration of action without development of tolerance. Granik and Grigor (12) proposed the mechanism for the release of NO from 1,2,5-oxadiazole *N*-oxides. It is also reported that release of NO from a nitric oxide donor drug produces beneficial effects such as reduction in blood pressure and prevention of atherosclerosis (11). NSAIDs possessing nitric oxide releasing capabilities are considered to be more promising drugs than the coxibs as these would be devoid of potential cardiovascular side effects associated with coxibs (11). Recently, a report has been published discussing the synthesis of some monosubstituted 3,4-diaryl-1,2,5-oxadiazoles (**11**) and *N*-oxides (**35**) as selective COX-2 inhibitors, expecting them to be free from adverse cardiovascular effects (13), but we claimed synthesis of these compounds much earlier (10).

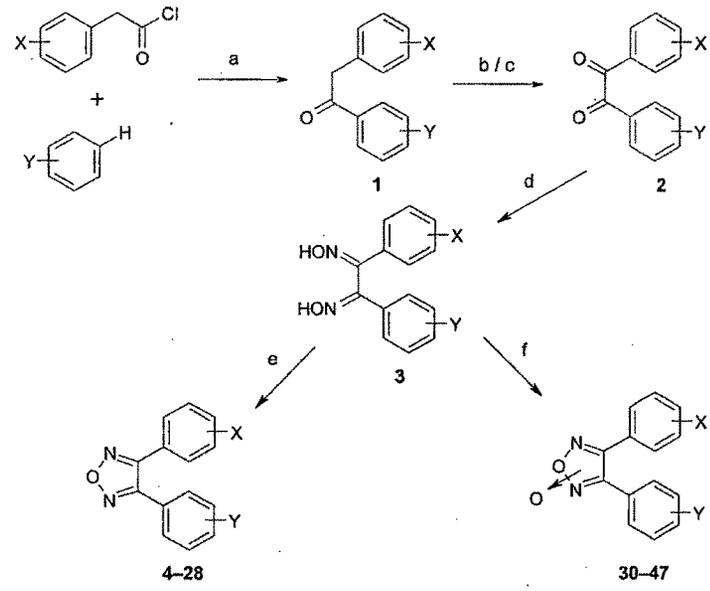
EXPERIMENTAL

The yields reported here are un-optimized. Melting points were determined using a heating block-type melting point apparatus and are uncorrected. The IR spectra were recorded using the KBr disc method on an FT-IR model 8300 (Shimadzu, Japan). The ^{1}H NMR spectra on a 300 MHz spectrometer (Brucker, USA) were recorded in $CDCl_3$ (chemical shifts in δ ppm). Assignment of exchangeable protons (NH) was confirmed by the D_2O exchange. Selenium dioxide oxidations were carried out in an R-330F microwave oven (Sharp, Carousel, Thailand). Final compounds were purified by passing through a silica gel H (100-200 mesh, s. d. fine chemicals, India) purifying column using a mixture of ethyl acetate and petroleum ether or chloroform alone as eluents.

Synthetic pathway is presented in Schemes 1 and 2, and physicochemical and spectral data for the synthesized compounds are given in Tables I and II.

The starting compounds, 1,2-diaryl-1,2-ethanediones (**2**) (benzils), were prepared by two routes. The first route involved benzoin condensation followed by oxidation (14, 15) while the second involved Friedel-Crafts acylation followed by selenium dioxide oxidation (16).

Syntheses of 1,2-diaryl-1,2-ethanedione dioximes (**3**). *General procedure.* – A mixture of 1,2-diaryl-1,2-ethanediones (**2**) (benzils) (10 mmol), hydroxylamine hydrochloride (60

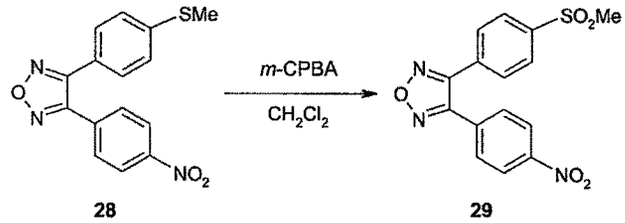


X = H, 2-Cl, 4-Cl, 4-F, 4-Me, 4-OMe, NO_2

Y = H, 4-Cl, 4-Br, 4-F, 4-Me, 4-OMe, 4-Sme, 4-SO_2Me, 3,4-di-OMe

Reagents and conditions: a – $AlCl_3$, CH_2Cl_2, 0–60 °C, 3–4 h; b – SeO_2, Ac_2O, reflux, 1–8 h; c – SeO_2, DMSO, microwave irradiation, 30–90 s; d – $NH_2OH \times HCl$, C_6H_5N, reflux, 7–8 h; e – $(\text{-}CH_2CO)_2O$, 180–5 °C, 10 min; f – aq. NaOCl (20%), 5–20 °C, 14–16 h

Scheme 1



Scheme 2

mmol) and pyridine (10 mL) was refluxed on an oil bath for 7 h. The reaction mixture was poured onto crushed ice containing concentrated hydrochloric acid (10 mL). The precipitate obtained was filtered, washed with cold water and dried. The crude materials were used as such for the next step without further purification.

Table I. Characterization data of 3,4-diaryl-1,2,5-oxadiazoles (4–29)

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
4	H	H	93–96[a]	49	$C_{14}H_{10}N_2O$ (222.25) 223 (M+H)	–	–	–	1577 (C=N-O), 1442 (N-O)	7.56–7.37 (m, 10H, ArH)
5	H	4′-Cl	83–84	21	$C_{14}H_9ClN_2O$ (256.69) 256 (M^+)	65.38 65.51	3.74 3.53	10.67 10.91	1597 (C=N-O), 1447 (N-O)	7.62–7.35 (m, 9H, ArH)
6	H	4′-Br	111–113	28	$C_{14}H_9BrN_2O$ (301.14) 302 (M+H)	56.12 55.84	2.79 3.01	9.48 9.30	1596 (C=N-O), 1446 (N-O)	7.59–7.39 (m, 9H, ArH)
7	H	4′-F	85–87	33	$C_{14}H_9FN_2O$ (240.24) 240 (M^+)	69.88 70.00	3.53 3.78	11.87 11.66	1605 (C=N-O), 1454 (N-O)	7.56–7.40 (m, 7H, ArH), 7.15–7.09 (m, 2H, 3′,5′-ArH)
8	H	4′-Me	57–59	30	$C_{15}H_{12}N_2O$ (236.28) 236 (M^+)	75.96 76.25	4.87 5.12	11.59 11.86	1605 (C=N-O), 1450 (N-O)	7.54–7.20 (m, 9H, ArH), 2.40 (s, 3H, $ArCH_3$)
9	H	4′-OMe	67–69	36	$C_{15}H_{12}N_2O_2$ (252.28) 252 (M^+)	71.77 71.42	4.64 4.79	10.97 11.10	1613 (C=N-O), 1456 (N-O), 1250 (Ar-O-Me, asym), 1025 (Ar-O-Me, sym)	7.55–7.37 (m, 7H, ArH), 6.91–6.88 (m, 2H, 3′,5′-ArH), 3.82 (s, 3H, $ArOCH_3$)
10	H	4′-SMe	83–84	27	$C_{15}H_{12}N_2OS$ (268.34) 269 (M+H)	67.47 67.14	4.35 4.51	10.62 10.44	1602 (C=N-O), 1435 (N-O)	7.54–7.40 (m, 7H, ArH), 7.26–7.23 (m, 2H, 3′,5′-ArH), 2.50 (s, 3H, $ArSCH_3$)
11	H	4′-SO_2Me	142–143[b]	47	$C_{15}H_{12}N_2O_3S$ (300.34)	60.26 59.99	4.32 4.03	9.16 9.33	1600 (C=N-O), 1448 (N-O), 1308 (SO_2 asym), 1149 (SO_2 sym)	8.03–8.00 (m, 2H, 3′,5′-ArH), 7.78–7.75 (m, 2H, 2′,6′ArH), 7.57–7.44 (m, 5H, ArH), 3.11 (s, 3H, $ArSO_2CH_3$)

Table I. Continued

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
12	2-Cl	H	59–60	26	$C_{14}H_9ClN_2O$ (256.69)	65.06 65.51	4.87 4.53	10.74 10.94	1600 (C=N-O), 1434 (N-O)	7.51–7.31 (m, 9H, ArH)
13	2-Cl	4′-Me	117–118	38	$C_{15}H_{11}ClN_2O$ (270.72)	66.90 66.55	3.79 4.10	10.55 10.35	1613 (C=N-O), 1435 (N-O)	7.51–7.39 (m, 4H, ArH), 7.37–7.34 (m, 2H, 2′,6′-ArH), 7.16–7.13 (m, 2H, 3′,5′-ArH), 2.35 (s, 3H, $ArCH_3$)
14	2-Cl	4′-OMe	107–108	39	$C_{15}H_{11}ClN_2O_2$ (286.72) 287 (M^+)	62.48 62.84	3.50 3.87	9.99 9.77	1611 (C=N-O), 1437 (N-O), 1253 (Ar-O-Me, asym), 1029 (Ar-O-Me, sym)	7.53–7.39 (m, 6H, ArH), 6.88–6.83 (m, 2H, 3′,5′-ArH), 3.80 (s, 3H, $ArOCH_3$)
15	2-Cl	3′,4′-di-OMe	77–79	60	$C_{16}H_{13}ClN_2O_3$ (316.75) 316 (M^+)	60.48 60.67	3.86 4.14	9.12 8.84	1605 (C=N-O), 1440 (N-O), 1256 (Ar-O-Me, asym), 1019 (Ar-O-Me, sym)	7.52–7.43 (m, 4H, ArH), 7.08–7.087 (dd, 1H, 2′-ArH), 7.02–6.98 (dd, 1H, 6′-ArH), 6.81–6.78 (dd, 1H, 3′-ArH), 3.87 (s, 3H, $ArOCH_3$), 3.71 (s, 3H, $ArOCH_3$)
16	4-Cl	4′-F	101–103	27	$C_{14}H_8FClN_2O$ (274.68)	60.82 61.22	3.22 2.94	10.38 10.20	1601 (C=N-O), 1447 (N-O)	7.54–7.48 (m, 2H, 2′,6′-ArH), 7.46–7.41 (m, 4H, ArH), 7.19–7.11 (m, 2H, 3′,5′-ArH)
17	4-Cl	4′-Me	137–139	19	$C_{15}H_{11}ClN_2O$ (270.72)	66.91 66.55	4.26 4.10	10.53 10.35	1602 (C=N-O), 1445 (N-O)	7.50–7.41 (m, 4H, ArH), 7.37–7.34 (m, 2H, 2′,6′-ArH), 7.16–7.13 (m, 2H, 3′,5′-ArH), 2.46 (s, 3H, $ArCH_3$)
18	4-Cl	4′-OMe	98–100	26	$C_{15}H_{11}ClN_2O_2$ (286.72)	62.57 62.84	4.28 3.87	9.95 9.77	1614 (C=N-O), 1451 (N-O), 1253 (Ar-O-Me, asym), 1027 (Ar-O-Me, sym)	7.51–7.40 (m, 6H, ArH), 6.98–6.93 (m, 2H, 3′,5′-ArH), 3.86 (s, 3H, $ArOCH_3$)

Table I. Continued

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
19	4-F	4′-SO_2Me	155–157	32	$C_{15}H_{11}FN_2O_3S$ (318.33)	56.33 56.60	3.86 3.48	8.56 8.80	1606 (C=N-O), 1310 (SO_2 asym), 1151 (SO_2 sym)	8.05–8.02 (m, 2H, 3′,5′-ArH), 7.77–7.74 (m, 2H, 2′,6′ArH), 7.53–7.49 (m, 2H, 2,6-ArH), 7.20–7.14 (m, 2H, 3,5-ArH), 3.12 (s, 3H, $ArSO_2CH_3$)
20	4-Me	4′-SO_2Me	141–142	43	$C_{16}H_{14}N_2O_3S$ (314.37)	61.48 61.13	4.24 4.49	9.02 8.91	1610 (C=N-O), 1451 (N-O), 1307 (SO_2 asym),1150 (SO_2 sym)	8.03–8.00 (m, 2H, 3′,5′-ArH), 7.78–7.75 (m, 2H, 2′,6′-ArH), 7.39–7.35 (m, 2H, 2,6-ArH), 7.28–7.25 (m, 2H, 3,5-ArH), 3.10 (s, 3H, $ArSO_2CH_3$), 2.43 (s, 3H, $ArCH_3$)
21	4-OMe	4′-OMe	123–125	26	$C_{16}H_{14}N_2O_3$ (282.30) 283 (M+H)	67.83 68.08	4.66 5.00	10.16 9.92	1612 (C=N-O), 1444 (N-O), 1257 (Ar-O-Me, asym), 1026 (Ar-O-Me, sym)	7.53–7.45 (m, 4H, ArH), 6.97–6.92 (m, 4H, 3,3′,5,5′-ArH), 3.86 (s, 6H, $ArOCH_3$)
22	4-NO_2	H	139–142	22	$C_{14}H_9N_3O_3$ (267.25) 267 (M^+)	63.24 62.92	3.02 3.39	15.58 15.72	1602 (C=N–O), 1515 (NO_2, asym), 1448 (N–O), 1350 (NO_2, sym)	8.31–8.28 (m, 2H, 3,5-ArH), 7.78–7.74 (m, 2H, 2,6-ArH), 7.58–7.45 (m, 5H, ArH)
23	4-NO_2	4′-Cl	168–170	53	$C_{14}H_8ClN_3O_3$ (301.69)	55.52 55.74	2.98 2.67	13.77 13.93	1601 (C=N–O), 1515 (NO_2, asym), 1448 (N–O) and 1350 (NO_2, sym)	8.34–8.31 (m, 2H, 3,5-ArH), 7.77–7.73 (m, 2H, 2,6-ArH), 7.50–7.43 (m, 4H, ArH)
24	4-NO_2	4′-Br	185–186	30	$C_{14}H_8BrN_3O_3$ (346.14)	48.16 48.58	2.66 2.33	12.38 12.14	1600 (C=N–O), 1517 (NO_2, asym), 1442 (N–O) and 1346 cm^{-1} (NO_2, sym)	8.34–8.30 (m, 2H, 3,5-ArH), 7.77–7.72 (m, 2H, 2,6-ArH), 7.65–7.60 (m, 2H, 2′,6′- ArH), 7.40–7.36 (m, 2H, 3′,5′-ArH)

Table I. Continued

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
25	4-NO_2	4′-F	137–138	46	$C_{14}H_8FN_3O_3$ (285.24)	58.55 58.95	2.59 2.83	14.59 14.73	1608 (C=N–O), 1519 (NO_2, asym), 1448 (N–O) and 1350 (NO_2, sym)	8.33–8.30 (m, 2H, 3,5-ArH), 7.76–7.73 (m, 2H, 2,6-ArH), 7.53–7.49 (m, 2H, 2′,6′-ArH), 7.21–7.15 (m, 2H, 3′,5′-ArH)
26	4-NO_2	4′-Me	113–114	39	$C_{15}H_{11}N_3O_3$ (281.27)	64.46 64.05	3.66 3.94	15.19 14.94	1602 (C=N–O), 1517 (NO_2, asym), 1448 (N–O) and 1346 cm^{-1} (NO_2, sym)	8.32–8.27 (m, 2H, 3,5-ArH), 7.78–7.74 (m, 2H, 2,6-ArH), 7.39–7.37 (m, 4H, ArH), 2.43 (s, 3H, $ArCH_3$)
27	4-NO_2	4′-OMe	125–126	32	$C_{15}H_{11}N_3O_4$ (297.27)	60.95 60.61	3.51 3.73	13.98 14.14	1610 (C=N–O), 1519 (NO_2, asym), 1436 (N–O), 1350 (NO_2, sym), 1249 (Ar–O) and 1024 cm^{-1} (O–Me)	8.31–8.28 (m, 2H, 3,5-ArH), 7.78–7.75 (m, 2H, 2,6-ArH), 7.45–7.41 (m, 2H, 2′,6′-ArH), 6.99–6.95 (m, 2H, 3′,5′-ArH), 3.87 (s, 3H, $ArOCH_3$)
28	4-NO_2	4′-SMe	135–136	34	$C_{15}H_{11}N_3O_3S$ (313.34)	57.12 57.50	3.85 3.54	13.28 13.41	1598 (C=N–O), 1519 (NO_2, asym), 1438 (N–O), 1350 cm^{-1} (NO_2, sym)	8.33–8.29 (m, 2H, 3,5-ArH), 7.79–7.74 (m, 2H, 2,6-ArH), 7.43–7.39 (m, 2H, 2′,6′-ArH), 7.31–7.27 (m, 2H, 3′,5′-ArH), 2.53 (s, 3H, $ArSCH_3$)
29	4-NO_2	4′-SO_2Me	172–173	59	$C_{15}H_{11}N_3O_5S$ (345.34)	51.83 52.17	3.52 3.21	12.34 12.17	1531 (NO_2 asym), 1353 (NO_2 sym), 1302 (SO_2 asym), 1149 cm^{-1} (SO_2 sym)	8.36–8.33 (m, 2H, 3,5-ArH), 8.09–8.06 (m, 2H, 3′,5′-ArH), 7.77–7.73 (m, 4H, ArH), 3.13 (s, 3H, $ArSO_2CH_3$)

[a] Lit. m.p. 94 °C (22), 67–70 °C (13).
[b] Lit. m.p. 135–137 °C (13).

Table II. Characterization data of 3,4-diaryl-1,2,5-oxadiazole N*-oxides (30–47)*

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
30	H	H	114–117[a]	65	$C_{14}H_{10}N_2O_2$ (238.25)	–	–	–	1592 (C=N^+-O^-), 1419 (=N^+(O^-)-O)	7.55–7.35 (m, 10H, ArH)
31	H	4′-Cl	104–105	33	$C_{14}H_9ClN_2O_2$ (272.69)	61.35 61.66	3.59 3.33	10.54 10.27	1591 (C=N^+-O^-), 1434 (=N^+(O^-)-O)	7.56–7.39 (m, 9H, ArH)
32	H	4′-F	113–115	30	$C_{14}H_9FN_2O_2$ (256.24) 256 (M^+)	65.47 65.62	3.79 3.54	11.12 10.93	1588 (C=N^+-O^-), 1429 (=N^+(O^-)-O)	7.56–7.40 (m, 7H, ArH), 7.17–7.08 (m, 2H, 3′,5′-ArH)
33	H	4′-Me	104–106	53	$C_{15}H_{12}N_2O_2$ (252.28) 252 (M^+)	71.09 71.42	4.48 4.79	11.35 11.10	1600 (C=N^+-O^-), 1429 (=N^+(O^-)-O)	7.54–7.38 (m, 7H, ArH), 7.25–7.18 (m, 2H, 3′,5′-ArH), 2.42 and 2.40 (s, 3H, $ArCH_3$)
34	H	4′-OMe	103–105	49	$C_{15}H_{12}N_2O_3$ (268.27)	67.42 67.16	4.82 4.51	10.57 10.44	1591 (C=N^+-O^-), 1425 (=N^+(O^-)-O), 1252(Ar-O-Me, asym), 1026 (Ar-O-Me, sym)	7.54–7.40 (m, 7H, ArH), 6.94–6.89 (m, 2H, 3′,5′-ArH), 3.84 and 3.83 (s, 3H, $ArOCH_3$)
35	H	4′-SO_2Me	125–127[b]	35	$C_{15}H_{12}N_2O_4$ (316.34) 316 (M^+)	57.33 56.95	3.67 3.82	8.67 8.86	1594 (C=N^+-O^-), 1448 (=N^+(O^-)-O), 1307 (SO_2 asym), 1150 cm^{-1} (SO_2 sym)	8.03–8.00 (m, 2H, 3′,5′-ArH), 7.79–7.74 (m, 2H, 2′,6′-ArH), 7.58–7.44 (m, 5H, ArH), 3.11 and 3.09 (s, 3H, $ArSO_2CH_3$)
36	2-Cl	4′-Me	139–140	66	$C_{15}H_{11}ClN_2O_2$ (286.72)	62.66 62.84	3.47 3.87	9.52 9.77	1592 (C=N^+-O^-), 1423 (=N^+(O^-)-O)	7.56–7.11 (m, 8H, ArH), 2.36 and 2.34 (s, 3H, $ArCH_3$)
37	2-Cl	4′-OMe	114–117	53	$C_{15}H_{11}ClN_2O_3$ (302.72)	59.91 59.52	3.82 3.66	9.07 9.25	1590 (C=N^+-O^-), 1426 (=N^+(O^-)-O), 1253 (Ar-O-Me, asym), 1029 (Ar-O-Me, sym)	7.57–7.37 (m, 6H, ArH), 6.88–6.84 (m, 2H, 3′,5′-ArH), 3.81 and 3.80 (s, 3H, $ArOCH_3$)

Table II. Continued

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (m/z)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
38	2-Cl	3′,4′-di-OMe	110–111	35	$C_{16}H_{13}ClN_2O4$ (332.75)	57.51 57.76	3.62 3.94	8.28 8.40	1601 (C=N$^+$-O$^-$), 1438 (=N$^+$(O$^-$)-O), 1264 (Ar-O-Me, asym), 1025 (Ar-O-Me, sym)	7.59–7.42 (m, 4H, ArH), 7.17–7.14 (m, 1H, 2′-ArH), 7.07–7.04 (m, 1H, 6′-ArH), 6.84–6.78 (m, 1H, 3′-ArH), 3.87, 3.72 and 3.63 (s, 6H, $ArOCH_3$)
39	4-Cl	4′-F	113–115	52	$C_{14}H_8ClFN_2O_2$ (290.68) 290 (M^+)	57.41 57.85	3.02 2.77	9.82 9.64	1605 (C=N$^+$-O$^-$), 1440 (=N$^+$(O$^-$)-O)	7.54–7.41 (m, 6H, ArH), 7.19–7.13 (m, 2H, 3′,5′-ArH)
40	4-Cl	4′-Me	121–123	37	$C_{15}H_{11}ClN_2O_2$ (286.72) 286 (M^+)	62.56 62.84	3.59 3.87	9.59 9.77	1589 (C=N$^+$-O$^-$), 1436 (=N$^+$(O$^-$)-O)	7.50–7.37 (m, 6H, ArH), 7.27–7.24 (m, 2H, 3′,5′-ArH), 2.42 and 2.40 (s, 3H, $ArCH_3$)
41	4-Cl	4′-OMe	142–144	53	$C_{15}H_{11}ClN_2O_3$ (302.72)	59.88 59.52	3.87 3.66	9.42 9.25	1591 (C=N$^+$-O$^-$), 1450 (=N$^+$(O$^-$)-O), 1254 (Ar-O-Me, asym), 1025 (Ar-O-Me, sym)	7.51–7.40 (m, 6H, ArH), 6.97–6.94 (m, 2H, 3′,5′-ArH), 3.86 and 3.85 (s, 3H, $ArOCH_3$)
42	4-OMe	4′-OMe	110–114	56	$C_{16}H_{14}N_2O_4$ (298.30) 299 (M+H)	64.66 64.42	4.99 4.73	9.17 9.39	1600 (C=N$^+$-O$^-$), 1443 (=N$^+$(O$^-$)-O)1261 (Ar-O-Me, asym), 1022 (Ar-O-Me, sym)	7.50–7.43 (m, 4H, ArH), 6.97–6.92 (m, 4H, 3′,5′-ArH), 3.85 and 3.84 (s, 6H, $ArOCH_3$)
43	4-NO_2	4′-Cl	156–158	78	$C_{14}H_8ClN_3O_4$ (317.69)	52.59 52.93	2.72 2.54	13.42 13.23	1591 (C=N$^+$–O$^-$), 1516 (NO_2, asym), 1440 (=N$^+$(O$^-$)–O), 1350 (NO_2, sym)	8.35–8.30 (m, 2H, 3,5-ArH), 7.77–7.71 (m, 2H, 2,6-ArH), 7.50–7.42 (m, 4H, ArH)

Table II. Continued

Compd. No.	X	Y	M.p. (°C)	Yield (%)	Molecular formula (M_r) and mass (*m/z*)	Elemental analysis, found/calcd. (%)			IR (ν, cm^{-1})	^1H NMR (δ, ppm)
						C	H	N		
44	4-NO_2	4′-Br	170–172	30	$C_{14}H_8BrN_3O_4$ (362.14)	46.22 46.43	2.49 2.23	11.45 11.60	1585 (C=N^+–O^-), 1521 (NO_2, asym), 1444 (=N^+(O^-)–O), 1350 cm^{-1} (NO_2, sym)	8.34–8.29 (m, 2H, 3,5-ArH), 7.77–7.71 (m, 2H, 2,6-ArH), 7.65–7.60 (m, 2H, 2′,6′-ArH), 7.40–7.35 (m, 2H, 3′,5′-ArH)
45	4-NO_2	4′-F	127–128	26	$C_{14}H_8FN_3O_4$ (301.24)	55.66 55.82	2.42 2.68	14.22 13.95	1589 (C=N^+–O^-), 1519 (NO_2, asym), 1440 (=N^+(O^-)–O), 1350 cm^{-1} (NO_2, sym)	8.34–8.29 (m, 2H, 3,5-ArH), 7.77–7.72 (m, 2H, 2,6-ArH), 7.53–7.48 (m, 2H, 2′,6′-ArH), 7.21–7.13 (m, 2H, 3′,5′-ArH)
46	4-NO_2	4′-Me	146–148	80	$C_{15}H_{11}N_3O_4$ (297.27)	60.27 60.61	3.97 3.73	14.29 14.14	1586 (C=N^+–O^-), 1540 (NO_2, asym), 1439 (=N^+(O^-)–O), 1350 (NO_2, sym)	8.31–8.26 (m, 2H, 3,5-ArH), 7.78–7.72 (m, 2H, 2,6-ArH), 7.39–7.35 (m, 4H, ArH), 2.44 and 2.42 (s, 3H, $ArCH_3$)
47	4-NO_2	4′-SO_2Me	156–158	46	$C_{15}H_{11}N_3O_6S$ (361.34)	49.69 49.84	2.78 3.06	11.49 11.62	1600 (C=N^+–O^-), 1523 (NO_2, asym), 1444 (=N^+(O^-)–O), 1350 (NO_2, sym), 1311 (SO_2, asym), 1153 cm^{-1} (SO_2, sym)	8.37–8.31 (m, 2H, 3,5-ArH), 8.10–8.04 (m, 2H, 3′,5′-ArH), 7.76–7.71 (m, 4H, ArH), 3.13 and 3.11 (s, 3H, $ArSO_2CH_3$)

[a] Lit. m.p 118 °C (22), Lit. 104–105 °C (13).
[b] Lit. m.p. 121–123 °C (13).

Syntheses of 3,4-diaryl-1,2,5-oxadiazoles (**4–28**). *General procedure.* – A mixture of 1,2-diaryl-1,2-ethanedione dioximes (**3**) (4 mmol) and succinic anhydride (20 mmol) was heated at 180–185 °C for 10 min in an oil-bath. The molten product was cooled, suspended in water and a sufficient quantity of sodium bicarbonate was added to neutralize the acid. The resulting mixture was extracted with successive quantities of chloroform (3 × 25 mL). The combined organic extract was washed with water (3 × 50 mL), dried and the solvent was recovered. The product obtained was crystallized from methanol to yield the title compounds

Preparation of 3-(4-methylsulfonylphenyl)-4-(4-nitrophenyl)-1,2,5-oxadiazole (**29**). – To a cooled solution of **28** (0.2 g, 0.64 mmol) in CH_2Cl_2 (20 mL), *m*-chloroperbenzoic acid (*m*-CPBA) (1.0 g, 58 mmol, 55–75%) was added under stirring. Stirring was continued at room temperature overnight. The reaction mixture was cooled to 0 °C and filtered at the pump to remove benzoic acid. Aqueous sodium metabisulphite (20 mL, 10%, *m*/*V*) was added to the filtrate and stirred for 15 min. The CH_2Cl_2 layer was separated and the aqueous layer was extracted with CH_2Cl_2 (3 × 5 mL). The combined organic extract was washed with aqueous sodium bicarbonate solution (5%, 2 × 10 mL) followed by water (3 × 5 mL). The CH_2Cl_2 layer was dried over anhydrous sodium sulphate and recovered. The resulting solid was crystallized from benzene to yield the title compound.

Syntheses of 3,4-diaryl-1,2,5-oxadiazole N*-oxides* (**30–47**). *General procedure.* – 1,2-Diaryl-1,2-ethanedione dioxime (**3**) (0.5 g) was dissolved in methanol (10 mL). A freshly prepared sodium hypochlorite solution (10 mL, 20%) was added dropwise to the above solution at a temperature below 10 °C under stirring over a period of 30 min keeping the temperature below 10 °C. After complete addition, the reaction mixture was further stirred for 1 h at a temperature below 10 °C and refrigerated overnight. The reaction mixture was poured onto crushed ice and the solid obtained was filtered and dried. Crystallization from methanol afforded the title compounds.

In vitro *COX inhibition assay*

The final compounds were evaluated for their ability to inhibit ovine COX-1 and COX-2 enzymes [percent inhibition at a fixed molar concentration (μmol L^{-1})] (17). Inhibition of the enzymes was determined with the colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemicals, USA) using ELISA reader following the procedure described in the catalog. The experiments were performed in duplicates.

In vivo *carrageenian induced rat paw edema assay*

Anti-inflammatory activity was determined by the carrageenian-induced rat paw edema method described by Winter *et al.* (18). Male Sprague-Dawley rats weighing 150 to 200 g (6–8 weeks old) were used in groups of six animals per group for the experiments. The animals were housed in a room with temperature of 22 ± 2 °C under a 12 h light/dark cycle. They were allowed free access to food and water *ad libitum*. The protocol for the animal experiments performed was approved by the IAEC (Institute Animal Ethics Committee) registered under CPCSEA (Committee for the purpose of Control and Supervision of Experiments on Animals) Govt. of India. Compounds were administered orally as suspension in 1% carboxymethyl cellulose (CMC). Paw edema was induced by

intradermal injection of 50 μL of 1% λ-carrageenan (Sigma, USA) into the subplantar region of the right hind paw, after one hour of compound administration. The paw volume was measured immediately after injection and after 3 hours using a plethysmometer (UGO-Basile, Italy). The control group received only the vehicle. Increase in paw volume was compared with that in the control group and percent inhibition was calculated taking the values in the control group as 0% inhibition.

Molecular modeling (docking studies)

All the molecular modeling studies reported herein were performed on a Silicon Graphics Fuel Workstation running on the IRIX 6.5 operating system using SYBYL 6.9 molecular modeling software from Tripos, Inc., USA (19) and GLIDE from Schrödinger Inc., USA (20). All compounds used for docking were built from the fragments in the SYBYL database. Each structure was fully geometry optimized using the standard Tripos force field (21) with a distance-dependent dielectric function until a root mean square deviation (rms) of 4.186 J $Å^{-1}$ was achieved. Conformational search was carried out using MULTISEARCH option in SYBYL 6.9. The lowest energy conformer thus obtained was further minimized using the Tripos force field and was subsequently used in docking. The COX-2 receptor structure (pdb code: 6COX) obtained from the Protein Data Bank (USA) was refined to remove water molecules, adjust bond orders and formal charges prior to docking. Docking was performed using GLIDE software according to their previously reported protocol (20).

RESULTS AND DISCUSSION

Chemistry

The general method employed for the preparation of 3,4-diaryl-1,2,5-oxadiazoles (**4–28**) and 3,4-diaryl-1,2,5-oxadiazole *N*-oxides (**30–47**) and important intermediates **1–3** is illustrated in Scheme 1. Acid chlorides of phenylacetic acid and substituted phenylacetic acids were obtained by refluxing the acid with thionyl chloride or phosphorous trichloride. Excess of thionyl chloride or phosphorous trichloride was removed under vacuum and the resulting acid chlorides were used as such in Friedel-Crafts acylation reaction with benzene and monosubstituted benzenes to yield 1,2-diaryl-1-ethanones (**1**) (16). IR spectra of these ethanones showed the presence of characteristic carbonyl stretching peaks at 1690–1665 cm^{-1}. Their ^1H NMR spectra showed characteristic signals for $-CH_2-$ at about δ 4.37 ppm.

1,2-Diaryl-1,2-ethanediones **2** (benzils) were synthesized by selenium dioxide (SeO_2) oxidation of **1** using AcOH/Ac_2O as solvents at refluxing temperatures up to 8 h. The reaction was completed under these conditions except for nitro substituted derivatives of **1**. Therefore, a new method was developed (16) for the oxidation of 1,2-diaryl-1-ethanones **1**, which proved to be faster and more efficient. In this method, the reaction was carried out in DMSO in a microwave oven for 30 s to afford the desired diones **2** in almost pure form. ^1H NMR spectra of these diones **2** showed the absence of characteristic signals for $-CH_2-$ at δ 4.37 ppm. Some of benzils **2** were prepared by benzoin/cross ben-

zoin condensation followed by oxidation, as per the reported procedures (14, 15). 1,2-Diphenyl-1,2-ethanedione (benzil), 1,2-di(4-methoxyphenyl)-1,2-ethanedione (anisil), 1-(2-chlorophenyl)-2-(4-methoxyphenyl)-1,2-ethanedione and 1-(2-chlorophenyl)-2-(3,4-dimethoxyphenyl)-1,2-ethanedione were prepared by this method (14, 15).

Benzils **2** were oximated into the corresponding 1,2-diaryl-1,2-ethanedione dioximes **3** using the hydroxylamine hydrochloride/pyridine system at refluxing temperatures. Most of the dioximes were isolated as solid compounds. Their TLC showed two spots and IR spectra indicated the absence of keto stretching bands. Since there is a possibility of formation of *syn* and *anti* products, no efforts were made to isolate these geometric isomers and the dioximes **3** were used as such for the next step.

Cyclization of **3** to 3,4-diaryl-1,2,5-oxadiazoles was attempted using different acidic/basic dehydrating agents but could only be effected by heating with succinic anhydride at 180–185 °C. Oxidation of **3** was carried out with aqueous sodium hypochlorite solution (20%) to obtain 3,4-diaryl-1,2,5-oxadiazole *N*-oxides. It was observed that methylsulfanyl (–SMe) also got oxidized to methylsulfonyl ($-SO_2Me$) during sodium hypochlorite treatment of compound **47**. This was confirmed by the shift of methyl signal from δ 2.50 to δ 3.11 ppm in its ^1H NMR spectrum. Conversion of –SMe to $-SO_2Me$ was also performed with *m*-CPBA either at 1,2-diaryl-1-ethanone **1** or at 3,4-diaryl-1,2,5-oxadiazole stages (Scheme 2). The elemental and spectral data of the synthesized compounds are given in Tables I and II.

Biological and molecular modeling studies

All compounds described herein were evaluated *in vitro* for COX-2 binding affinity at a concentration of 22 μmol L^{-1} by the colorimetric COX (ovine) inhibitor screening assay. Selected active compounds were also evaluated for COX-1 binding affinity at a higher concentration (88 μmol L^{-1}) (Table III). Compounds that showed promising COX-2 inhibitory activity were further screened for their anti-inflammatory activity (Table IV) *in vivo* using the carrageenian induced rat paw edema method.

Amongst all the compounds, methoxy (-OMe) substituted compounds **9**, **21**, **34**, **41**, **42** showed COX-2 enzyme inhibition higher than that shown by compounds with other substituents. 3,4-Di(4-methoxyphenyl)-1,2,5-oxadiazole *N*-oxide (**42**) showed COX-2 enzyme inhibition of 54% at 22 μmol L^{-1} and COX-1 enzyme inhibition of 44% at 88 μmol L^{-1} concentration, but showed mild *in vivo* anti-inflammatory activity at a 25 mg kg^{-1} dose. However, its deoxygenated analog **21** showed lower COX-2 enzyme inhibition (26% at 22 μmol L^{-1}) and higher COX-1 enzyme inhibition (53% at 88 μmol L^{-1}), but showed stronger *in vivo* anti-inflammatory activity at a 25 mg kg^{-1} dose (71%) than standard celecoxib at 12.5 mg kg^{-1} (48%). However, at the same dose level of 12.5 mg kg^{-1}, it showed much lower activity than (21%) celecoxib. This preliminary study suggests that the methoxy (-OMe) group at 4-position of one of the phenyl rings may be a suitable pharmacophore for COX-2 enzyme binding in this series of compounds. Replacement of one of the -OMe groups of compound **21** by an electron withdrawing $-NO_2$ group resulted in a complete loss of COX-2 enzyme affinity. Compounds **11**, **19**, **20**, **29** and **47** with the well known COX-2 enzyme pharmacophore (methylsulfonyl, $-SO_2Me$) failed to show COX-2 enzyme inhibition at a 22 μmol L^{-1} concentration, but compound **11** exhibited *in vivo* anti-inflammatory activity at 25 mg kg^{-1} comparable to celecoxib at 12.5 mg kg^{-1}. Compound **21** was found to be the most active compound in the series.

Table III. In vitro *COX-2 inhibition data for 3,4-diaryl-1,2,5-oxadiazoles and 3,4-diaryl-1,2,5-oxadiazole* N*-oxides (4–47)*[a]

Compd. No.	Inhibition[b] (%)	
	COX-2 (22 μmol L^{-1})	COX-1 (88 μmol L^{-1})
4	5	–
8	8	–
9	17	–
10	5	–
14	9	–
15	5	–
18	4	–
19	4	–
21	26	53
23	4	–
25	7	–
32	3	–
34	21	55
35	11	–
37	6	–
38	4	–
41	20	53
42	54	45
45	2	–
42	54	45
Celecoxib	95	–

[a] Compounds **5–7, 11–13, 16, 17, 20, 22, 24, 26–31, 33, 36, 39, 40, 43, 44, 46** and **47** were found to be inactive at the concentration of 22 μmol L^{-1}.
– Not evaluated.
[b] Experiments were performed in duplicate.

All the synthesized compounds were energy minimized and docked in the active site of COX-2, but only a few binding interactions are discussed here. The binding interaction of 3,4-di(4-methoxyphenyl)-1,2,5-oxadiazole (**21**, 26% COX-2 inhibitory activity) was studied within the COX-2 binding site by molecular docking studies. The *para* methoxy group is oriented in the vicinity of COX-2 secondary pocket (Phe^{518}, Arg^{513}, Gln^{192}, Val^{523}, Ser^{353}) as shown in Fig. 1. The oxygen atom of the *para* substituted methoxy group to the C-3 phenyl ring is hydrogen bonded with the backbone NH of Ile^{517} (distance = 4.1 Å). The oxygen atom of the other methoxy group that is *para* substituted to C-4 phenyl ring also forms a hydrogen bond with OH of Tyr^{348} (distance = 4.9 Å). The N^2-atom of the central oxadiazole ring forms a favorable hydrogen bond with NH_2 of Arg^{120} (distance = 3.65 Å).

Table IV. In vivo *anti-inflammatory data for selected compounds*

Compd. No.	Dose (mg kg^{-1})	Paw volume (mL) (% inhibition)[a]
Control	25	0.820 ± 0.02 (0)
11	25	0.28 ± 0.06 (53)
20	25	0.57 ± 0.10 (35)
21	25	0.16 ± 0.04 (71)
41	25	0.25 ± 0.03 (31)
42	25	0.04 ± 0.01 (5)
21	12.5	0.16 ± 0.04 (21)
Celecoxib	12.5	0.39 ± 0.05 (48)

[a] Mean ± SD, $n = 6$.

Binding mode of 3,4-di(4-methoxyphenyl)-1,2,5-oxadiazole *N*-oxide (**42**, 54% COX-2 inhibitory activity) was also examined (Fig. 2). As observed in other compounds, also here the *p*-methoxy group is oriented towards the secondary pocket of the enzyme, which is similar to the orientation of $-SO_2NH_2$ in celecoxib. The central oxygen atom of the oxadiazole ring participates in hydrogen bond formation with NH_2 of Arg^{120} (distance = 2.57 Å). Oxygen atom of *N*-oxide forms a favorable hydrogen bond with NH of Leu^{531}. The methoxy group substituted on the C-3 phenyl ring forms a hydrogen bond with Tyr^{348} (distance = 4.8 Å). The methoxy group on the C-4 phenyl ring also participates in the formation of the hydrogen bond with backbone NH of Ile^{517} (distance = 3.83 Å) and NH of Gln^{192} (distance = 4.8 Å). Amongst all the compounds in the series, **42** had the lowest intermolecular energy of -3.5×10^5 J mol^{-1}, indicating its stability in the COX-2 active site.

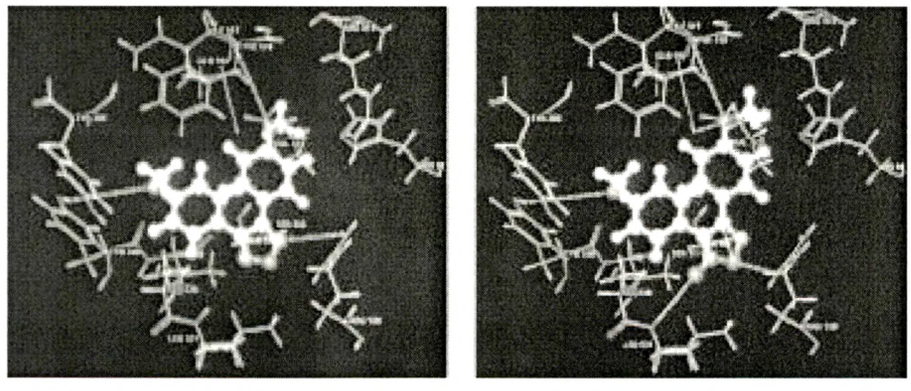


Fig. 1. Docking of compounds (ball and stick) in the active site of murine COX-2: a) 3,4-di(4-methoxyphenyl)-1,2,5-oxadiazole (**21**) and b) 3,4-di(4-methoxyphenyl)-1,2,5-oxadiazole *N*-oxide (**42**).

CONCLUSIONS

Docking studies of 3,4-diaryl-1,2,5-oxadiazoles and 3,4-diaryl-1,2,5-oxadiazole *N*-oxides indicate a favorable orientation of the methoxy group in the COX-2 active site. Lower binding energies also indicate the stability of 3,4-diaryl-1,2,5-oxadiazoles and their *N*-oxides in the active site. This supports *in vitro* and *in vivo* anti-inflammatory data. These preliminary studies suggest that the methoxy group may be acting as a pharmacophore for the COX-2 enzyme binding site in series of 1,2,5-oxadiazoles and their *N*-oxides. Further work in this direction is in progress.

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SAŽETAK

Istraživanja 3,4-diaril-1,2,5-oksadiazola i njihovih *N*-oksida: Potraga za boljim COX-2 inhibitorima

MANGE RAM YADAV, SHRIKANT T. SHIRUDE, DEVENDRA S. PUNTAMBEKAR, PINKAL J. PATEL, HETAL B. PRAJAPATI, ARVIND PARMAR, R. BALARAMAN i RAJANI GIRIDHAR

Sintetizirana je serija 3,4-diaril-1,2,5-oksadiazola i 3,4-diaril-1,2,5-oksadiazol *N*-oksida i ocijenjena njihova sposobnost vezivanja na COX-2 i COX-1 *in vitro* i protuupalno djelovanje na edem šape štakora. Spojevi sa *p*-metoksi (*p*-OMe) supstituentom **9**, **21**, **34**, **41**, **42** bolje su inhibirali COX-2 nego ostali spojevi. 3,4-*Di*(4-metoksifenil)-1,2,5-oksadia-

zol *N*-oksid (**42**) inhibirao je COX-2 za 54% u koncentraciji od 22 μmol L^{-1}, a COX-1 za 44% u koncentraciji 88 μmol L^{-1}, ali je *in vivo* slabo djelovao protuupalno. Njegov deoksigenirani derivat **21** pokazao je slabiju inhibiciju COX-2 enzima (26% u koncentraciji 22 mmol L^{-1}) i jaču inhibiciju COX-1 (71% u koncentraciji 25 mg kg^{-1}), što je bolje od standarda celokoksiba (48% u koncentraciji 12,5 mg kg^{-1}). Molekularno je modeliranje pokazalo da je metoksi skupina smještena u blizini sekundarnog džepa na enzimu COX-2 i da utječe na vodikove veze interakcija na aktivnom mjestu COX-2. Ova preliminarna istraživanja sugeriraju da bi se u seriji oksadiazol/*N*-oksida mogao naći predvodni spoj s *p*-metoksi skupinom na benzenskom prstenu.

Ključne riječi: 1,2,5-oksadiazol, 1,2,5-oksadiazol *N*-oksid, COX-2 inhibitor

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Effect of pioglitazone on L-NAME induced hypertension in diabetic rats

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Abstract

The present study investigates the effect of pioglitazone treatment on blood pressure, vascular reactivity and antioxidant enzymes in L-NAME induced hypertension in normal and STZ-diabetic rats.

Diabetes was induced in male Sprague Dawley rats (200±15 g) by single intravenous injection of 55 mg/kg of streptozotocin (STZ). Rats were randomized into diabetic and nondiabetic groups, Nω-nitro-L-arginine-methyl ester (L-NAME, 50 mg/kg) was administered in drinking water for 4 weeks. They were treated with pioglitazone (10 mg/kg/day, p.o.) for 4 weeks and following protocol was carried out. Blood pressure, blood glucose levels and body weight were measured. Thoracic aorta was isolated and dose response curve of phenylephrine (PE) with intact and denuded endothelium was recorded. Dose response curve of acetylcholine (Ach) and sodium nitroprusside (SNP) was recorded in precontracted rings. Lipid peroxidation, superoxide dismutase, catalase, and reduced glutathione were estimated in liver, kidney, and aorta.

Pioglitazone produced no significant effect on blood glucose levels, body weight and blood pressure of L-NAME administered nondiabetic and diabetic rats. Pioglitazone treatment had no significant effect on PE induced contraction and Ach induced relaxation in L-NAME diabetic and nondiabetic rats. SNP completely relaxed aortic rings of all the goups. Higher oxidative stress in case of diabetic rats was significantly ($p<0.05$) reduced by pioglitazone treatment.

Although pioglitazone reduced oxidative stress in diabetic rats, there was no significant effect on blood pressure as there was complete absence of nitric oxide due to administration of L-NAME. Hence from the present study it can be concluded that reduction in blood pressure in case of STZ-diabetic rats is nitric oxide mediated.

Keywords: Blood pressure; Diabetes; Nω-nitro-L-arginine-methyl ester; Pioglitazone; Streptozotocin

1. Introduction

Diabetes mellitus has been identified as a primary risk factor for cardiovascular diseases (Uemura et al., 2001) and alters vascular responsiveness to several vasoconstrictors and vasodilators (Senses et al., 2001). Most of the complications in diabetes are due to increased serum glucose and increased generation of oxygen-derived free radicals, which lead to endothelium dysfunction. This endothelium dependent vasodilation is reduced in diabetes largely due to excessive oxidative stress and bioavailability of nitric oxide.

Pioglitazone, a PPAR (peroxisome proliferators activated receptors) gamma agonist, improves insulin-mediated glucose uptake into skeletal muscle without increasing endogenous insulin secretion (Ikeda et al., 1990) and has been demonstrated to be effective in the treatment of non-insulin dependent diabetes mellitus with insulin resistance. Pioglitazone lowers blood pressure and restores blunted endothelium dependent vasodilatation in fructose fed rats (Kotchen et al., 1997), insulin-resistant Rhesus monkey (Kemnitz et al., 1994), SHR (Grinsell et al., 2000) and sucrose fed SHR (Uchida et al., 1997). Recently we have shown that pioglitazone restores endothelial function and reduces blood pressure in streptozotocin (STZ) induced diabetic rats (Majithiya et al., 2005). Moreover there are no reports of effect of pioglitazone in L-NAME hypertensive rats wherein nitric oxide is completely blocked. Hence the purpose of the present study was to investigate the effect of pioglitazone treatment, on blood pressure and aortic contractibility in L-NAME induced hypertension in normal and STZ-diabetic rats.

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2. Materials and methods

2.1. Drugs

Pioglitazone hydrochloride was obtained as a gift sample from Alembic Ltd, Baroda. Streptozotocin, phenylephrine, acetylcholine and *N*ω-nitro-L-arginine-methyl ester (L-NAME) were obtained from SIGMA, St. Louis, MO, USA. Sodium nitroprusside and sodium carboxy methylcellulose were obtained from S.D. Fine chemicals India ltd. All other chemicals and reagents used in the study were of analytical grade. Stock solution of phenylephrine, acetylcholine, sodium nitroprusside and L-NAME was prepared in double distilled water. For oral administration pioglitazone suspension was prepared in 0.5% sodium carboxy methyl cellulose.

2.2. Animals

All experiments and protocols described in the present report were approved by the Institutional Animal Ethics Committee (IAEC) of M. S. University, Baroda and are in accordance with guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Male Sprague Dawley rats (200±15 g) were housed in group of 3 animals and maintained under standardized condition (12-h light/dark cycle, 24 °C) and provided free access to pelleted food (Pranav Agro Pvt. Ltd.) and purified drinking water ad libitum. Diabetes was induced by single intravenous injection of 55 mg/kg of streptozotocin (STZ) dissolved in normal saline. The control animals were injected with equal volume of vehicle. After 3 days following streptozotocin administration, blood was collected from tail vein and serum samples were analyzed for blood glucose (Enzymatic kits, GOD/POD method, SPAN diagnostics Pvt. Ltd). Animals showing fasting blood glucose higher than 250 mg/dl were considered as diabetic rats and used for the study. Four weeks after induction of diabetes, blood pressure was measured by tail cuff method and rats with systolic blood pressure higher than 135 mm Hg were selected, randomized into groups and used for the study. Age matched nondiabetic rats with systolic blood pressure less than 120 mm Hg were randomized into nondiabetic groups. Diabetic ($n=30$) and nondiabetic rats ($n=30$) were divided into the following groups: nondiabetic control (ND-CON), STZ-diabetic control (STZ-CON), nondiabetic rats treated with L-NAME (50 mg/kg/day, in drinking water) for 4 weeks (ND-L-NAME), STZ-diabetic rats treated with L-NAME (50 mg/kg/day, in drinking water), nondiabetic rats treated with L-NAME (50 mg/kg/day, in drinking water) and pioglitazone (10 mg/kg/day) (ND-L-NAME+PIO) and STZ-diabetic rats treated with L-NAME (50 mg/kg/day, in drinking water) and pioglitazone (10 mg/kg/day) for 4 weeks (STZ-L-NAME+PIO). Respective control groups were orally administered with vehicle (1 ml/kg/day of 0.5% sodium carboxy methyl cellulose solution) for 4 weeks. Age matched nondiabetic rats were used in the study.

2.3. Blood pressure

Blood pressure was measured noninvasively at the start of study and at weekly intervals by tail cuff method using LE 5002 storage pressure meter (LETICA scientific instruments, SPAIN) in all the above mentioned groups. For the blood pressure measurements animals were trained for at least 1 week until blood pressure was steadily recorded with minimal stress and restraint. The mean of 7–8 measurements of trained animals was recorded.

2.4. Preparation of aortic rings

The thoracic aorta of rats was isolated immediately after decapitation and carefully cleaned of fat and connective tissues. The aorta was cut into rings of 3 mm width. Aortic rings were suspended between two 'S' shaped platinum loops in jacketed organ bath containing 20 ml Krebs bicarbonate solution (pH 7.4) maintained at 37±0.5 °C and continuously aerated with 95% oxygen and 5% carbon dioxide. The composition of the Krebs solution (mmol/L) was NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 22.0, and glucose 11.0. The rings were connected to isometric force displacement transducer connected to Gemini pen recorder (UGO-BASILE, Italy). The rings were maintained under tension of 2 *g* and equilibrated for 90 min before initiating experimental protocol. During this period, the Krebs solution was changed at every 15-min interval. After the equilibration period rings were maximally contracted with phenylephrine (PE, 1 μM) to test their contractile capacity, three recordings were carried out to find out a constant and reproducible contraction.

2.5. In vitro vascular reactivity on aortic rings of control and pioglitazone treated rats

After 4 weeks of treatment aortic rings of all the groups were isolated and mounted in organ bath as described above. Concentration response curves to increasing concentrations of PE (1 nM–10 μM) were performed in rings with endothelium. After generating PE contraction curves each ring was serially washed to baseline and equilibrated. Rings were precontracted submaximally with PE (which produced 80–90% of maximum response) to reach a stable plateau. Endothelium mediated

Table 1
Body weight and blood glucose level of ND-CON, STZ-CON, ND-L-NAME, STZ-L-NAME, ND-L-NAME+PIO and STZ-L-NAME+PIO groups

Groups	Body weight (g)		Blood glucose level (mg/dl)	
	Initial	Final	Initial	Final
ND-CON	207±12.4	251±14.2	89±4.5	93±6.7
STZ-CON	211±14.7	224±17.8	423±24.5[a]	441±31.7[a]
ND-L-NAME	208±11.2	247±9.3	88±5.7	94.6±4.2
STZ-L-NAME	207±17.5	221±16.2	431±21.6[a]	448±29.1[a]
ND-L-NAME+PIO	212±12.7	262±12.4	91±4.1	93±6.8
STZ-L-NAME+PIO	209±13.2	233±18.5	428±26.7[a]	440±34.2[a]

Values are expressed as mean±SEM ($n=5–8$).
[a] Significantly different from ND-CON group, $p<0.05$.

relaxation was measured as a concentration response curve to Ach (1 nM–10 μM). Relaxation to sodium nitroprusside (SNP, 0.001 nM–1 μM) was also measured as a concentration response curve in aortic rings.

2.6. Measurement of superoxide dismutase, catalase, reduced glutathione and lipid peroxidation

After 4 weeks of treatment animals were sacrificed. Liver, kidney and aorta were isolated and weighed (Bafna and Balaraman, 2004). The tissues were finely sliced and homogenized in chilled tris buffer at a concentration of 10% (w/v). The homogenates were centrifuged at 10,000 ×g at 0 °C for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for estimation for assays of lipid peroxidation (MDA content), endogenous antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) and reduced glutathione (GSH)). Superoxide dismutase was determined by the method of Mishra and Fridovich (1972). Catalase was estimated by the method of Hugo Aebi as given by Aebi (1984). Reduced glutathione was determined by the method of Moron et al. (1979). Lipid peroxidation or malondialdehyde

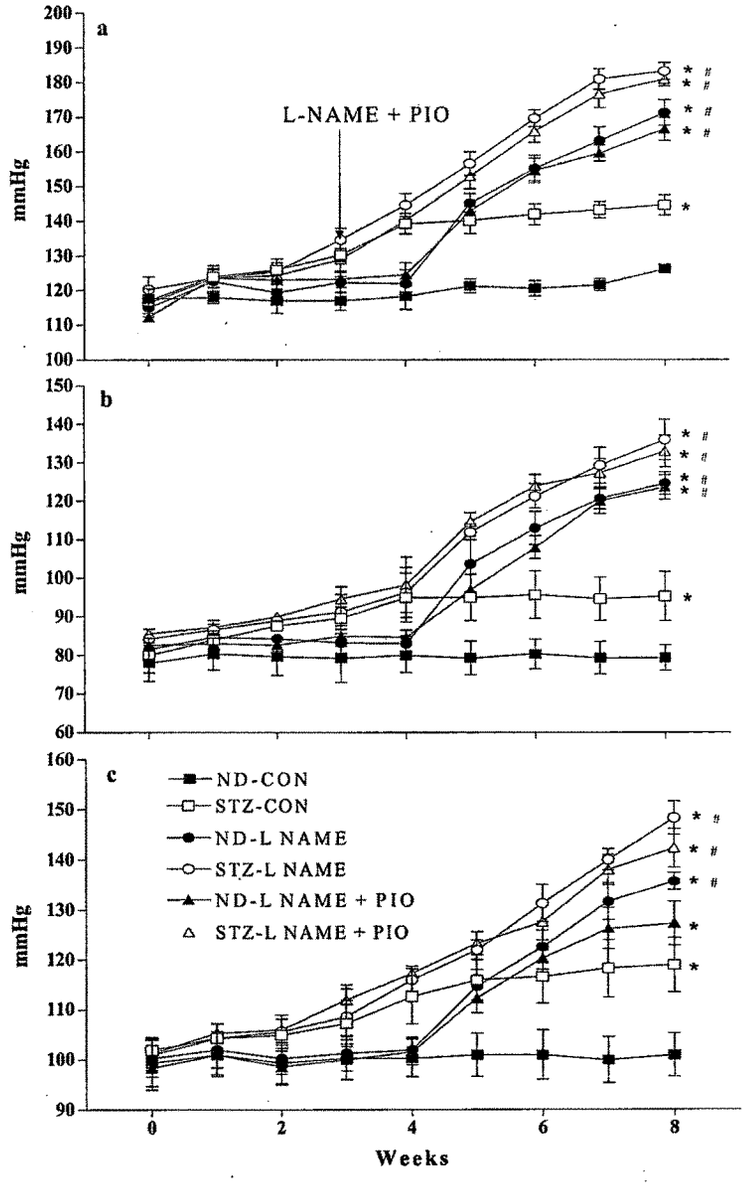

Fig. 1. Effect of pioglitazone treatment for 4 weeks in systolic (a), diastolic (b) and mean (c) blood pressure on ND-control (-•-), STZ-control (-□-), ND-L-NAME (-•-), STZ-L-NAME (-○-), ND-L-NAME+PIO (-▲-) and STZ-L-NAME+PIO (-Δ-) groups. All the values are expressed as mean±SEM. *Significantly different from ND-CON group, $p<0.05$. #Significantly different from STZ-CON group, $p<0.05$.

formation was estimated by the method of Slater and Sawyer (1971).

2.7. Statistical analysis

All the data are expressed as mean±SEM. Data were analyzed by ANOVA for repeated measurements followed by Bonferroni multiple comparison tests. Differences were considered to be statistically significant when $p<0.05$. The agonist pD_2 value ($-\log EC_{50}$) was calculated from concentration response curve by non-linear regression analysis of the curve using computer based fitting program (Prism, Graphpad).

3. Results

3.1. Body weight and blood glucose level

Body weight and blood glucose levels of all the groups are shown in Table 1. L-NAME treatment had no significant effect on body weight of nondiabetic or diabetic rats as compared to their respective control. Administration of pioglitazone produced no significant change in body weight of nondiabetic or diabetic animals as compared to respective controls. Serum blood glucose levels were significantly increased in all STZ administered animals. Treatment with pioglitazone produced no significant change in blood glucose levels.

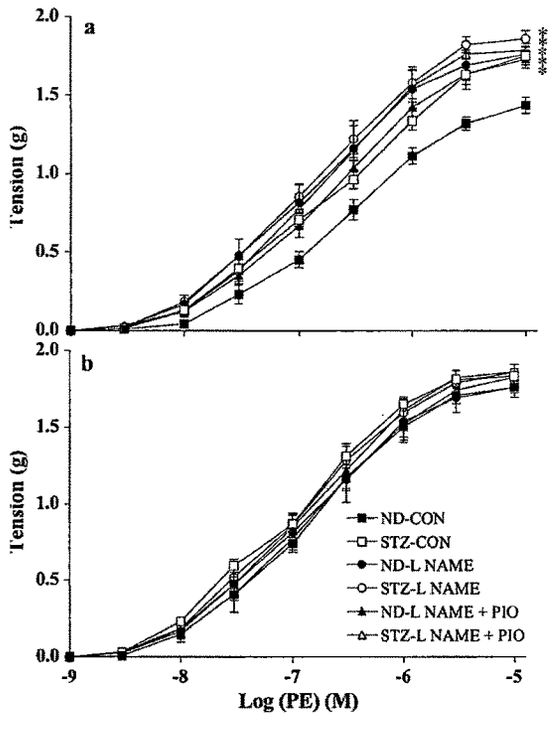

Fig. 2. Concentration response curve of phenylepherine on aortic rings with intact endothelium (a) or with denuded endothelium (b) of ND-control (-•-), STZ-control (-□-), ND-L-NAME (-•-), STZ-L-NAME (-°-), ND-L-NAME+PIO (-▲-) and STZ-L-NAME+PIO (-Δ-) groups. Values are expressed as mean±SEM. *Significantly different from ND-CON group, $p<0.05$. #Significantly different from STZ-CON group, $p<0.05$ ($n=5-8$).

Table 2
Maximal response (E_{max}) and pD_2 ($-\log EC_{50}$) values of phenylephrine in aortic rings with intact or denuded endothelium

Groups	Phenylephrine			
	With intact endothelium		With denuded endothelium	
	pD_2	E_{max} (g)	pD_2	E_{max} (g)
ND-CON	6.60±0.054	1.43±0.034	6.83±0.063	1.78±0.044
STZ-CON	6.71±0.068	1.70±0.048[a]	6.99±0.058	1.83±0.038
ND-L-NAME	6.92±0.087[a]	1.74±0.059[a]	6.92±0.087	1.77±0.054
STZ-L-NAME	6.89±0.069[a]	1.84±0.050[a]	6.95±0.062	1.83±0.044
ND-L-NAME+PIO	6.74±0.044	1.72±0.049[a]	6.89±0.065	1.85±0.048
STZ-L-NAME+PIO	6.83±0.051	1.80±0.037[a]	6.84±0.058	1.80±0.042

Values are expressed as mean±SEM ($n=5-8$).
[a] Significantly different from ND-CON group, $p<0.05$.

3.2. Blood pressure

Blood pressure was higher in case of diabetic animals as compared to nondiabetic animals and administration of L-NAME significantly increased blood pressure of all the groups (Fig. 1). There was a significant ($p<0.05$) increase in systolic (144±2.96), diastolic (97.5±6.16) and mean blood pressure (119±5.63) of STZ-CON group as compared to ND-control (Fig. 1). Administration of L-NAME significantly ($p<0.05$) increased systolic (183±4.71), diastolic (136.5±5.46) and mean blood pressure (149±3.33) of STZ-L-NAME group. There was no significant effect of pioglitazone treatment for 4 weeks on systolic, diastolic and mean blood pressure in both L-NAME treated groups i.e. STZ diabetic or nondiabetic groups.

3.3. Contraction response to PE

Cumulative addition of PE (1 nM–10 μM) to organ bath resulted in concentration dependent contraction of aortas of all groups (Fig. 2). There was a significant ($p<0.05$) increase in maximal response of PE in STZ-CON, ND-L-NAME, STZ-L-NAME groups as compared to ND-CON group (Fig. 2, Table 2). Pioglitazone treatment had no significant effect on maximal response to PE in ND-L-NAME+PIO and STZ-L-NAME+PIO group as compared to STZ-CON. pD_2 values of all the groups are shown in Table 2.

3.4. Ach and SNP induced relaxation responses in control and pioglitazone treated rats

Addition of Ach resulted in concentration dependent relaxation of aortic rings of ND-CON group. Ach induced relaxation in aortas of STZ-CON group was significantly ($p<0.05$) lower as compared to ND-CON group (Fig. 3). Ach induced relaxation was completely blocked in L-NAME administered groups. Ach induced relaxation in all the L-NAME administered groups was significantly ($p<0.05$) reduced as compared to ND-CON group and STZ-CON group. Pioglitazone (10 mg/kg/day) treatment had no significant change in Ach induced relaxation in STZ-L-NAME+PIO

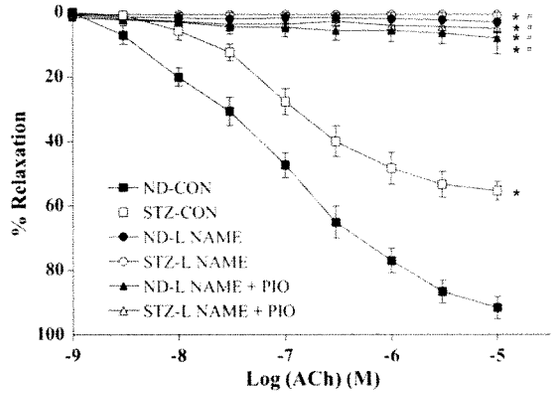

Fig. 3. Concentration dependent relaxation to acetylcholine on aortic rings of ND-control (-•-), STZ-control (-□-), ND-L-NAME (-•-), STZ-L-NAME (-○-), ND-L-NAME + PIO (-▲-) and STZ-L-NAME + PIO (-Δ-) groups. Values are expressed as mean ± SEM. *Significantly different from ND-CON group, $p < 0.05$. #Significantly different from STZ-CON group, $p < 0.05$ ($n = 5–8$). Tension is expressed as percent relaxation on initial contraction with PE.

group as compared to STZ-L-NAME group (Fig. 3). Addition of SNP completely relaxed aortic rings of all the groups. There was no significant change to SNP induced relaxation in aortic rings in any of the groups (Fig. 4).

3.5. Superoxide dismutase, catalase, reduced glutathione and lipid peroxidation

Administration of STZ significantly ($p < 0.05$) increased oxidative stress in liver, kidney and aorta of STZ-CON group as compared to ND-CON group (Table 3). SOD, CAT and GSH were significantly decreased while lipid peroxidation was significantly increased in all STZ administered group. Pioglitazone treatment significantly ($p < 0.05$) increased levels of endogenous antioxidants (SOD, CAT and GSH) in liver, kidney and aorta as compared to STZ-CON (Table 3). Moreover lipid peroxidation was significantly ($p < 0.05$) decreased in liver, kidney and aorta of STZ-L-NAME + PIO group as compared to STZ-CON (Table 3). There was no significant change in SOD, CAT, GSH and lipid peroxidation due to pioglitazone treatment in normal rats (ND-L-NAME) as compared to ND-CON.

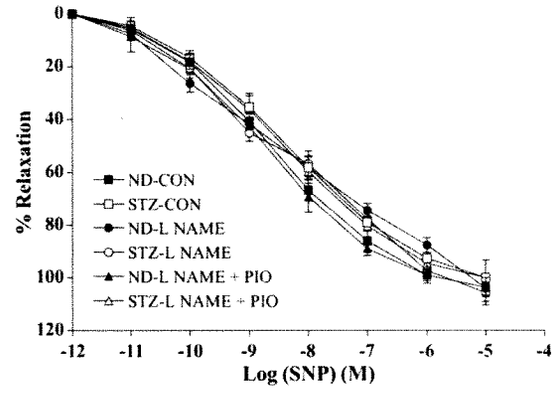

Fig. 4. Concentration dependent relaxation to sodium nitroprusside on aortic rings of ND-control (-•-), STZ-control (-□-), ND-L-NAME (-•-), STZ-L-NAME (-○-), ND-L-NAME + PIO (-▲-) and STZ-L-NAME + PIO (-Δ-) groups. Values are expressed as mean ± SEM. *Significantly different from ND-CON group, $p < 0.05$. #Significantly different from STZ-CON group, $p < 0.05$ ($n = 5–8$).

Table 3
Effect of pioglitazone treatment on superoxide dismutase, catalase, reduced glutathione and lipid peroxidation

Groups	Liver	Kidney	Aorta
Superoxide dismutase (U/mg protein)			
ND-CON	7.41 ± 0.212	9.53 ± 0.154	5.77 ± 0.088
STZ-CON	5.10 ± 0.098[a]	5.31 ± 0.139[a]	4.36 ± 0.066[a]
ND-L-NAME	7.15 ± 0.119	9.61 ± 0.210	5.89 ± 0.033
STZ-L-NAME	5.02 ± 0.137[a]	5.85 ± 0.147[a]	4.19 ± 0.380[a]
ND-L-NAME + PIO	6.97 ± 0.206	9.41 ± 0.085	5.70 ± 0.087
STZ-L-NAME + PIO	6.21 ± 0.214[b]	6.80 ± 0.203[b]	4.90 ± 0.144[b]
Catalase (μM of H_2O_2 consumed/min mg protein)			
ND-CON	11.44 ± 0.17	11.40 ± 0.50	5.88 ± 0.054
STZ-CON	6.80 ± 0.414[a]	6.12 ± 0.53[a]	3.61 ± 0.106[a]
ND-L-NAME	11.30 ± 0.211	11.28 ± 0.35	5.67 ± 0.114
STZ-L-NAME	6.45 ± 0.330[a]	6.21 ± 0.18[a]	3.55 ± 0.208[a]
ND-L-NAME + PIO	11.68 ± 0.335	11.69 ± 0.55	5.59 ± 0.180
STZ-L-NAME + PIO	8.33 ± 0.582[b]	7.90 ± 0.40[b]	4.66 ± 0.309[b]
Reduced glutathione (μg of GSH/mg protein)			
ND-CON	9.86 ± 0.182	11.44 ± 0.133	2.81 ± 0.181
STZ-CON	4.06 ± 0.022[a]	3.89 ± 0.334[a]	0.79 ± 0.047[a]
ND-L-NAME	9.70 ± 0.110	11.23 ± 0.310	2.88 ± 0.131
STZ-L-NAME	3.89 ± 0.077[a]	3.72 ± 0.095[a]	0.68 ± 0.033[a]
ND-L-NAME + PIO	9.64 ± 0.121	10.82 ± 0.260	2.72 ± 0.070
STZ-L-NAME + PIO	5.10 ± 0.220[b]	5.33 ± 0.287[b]	1.18 ± 0.065[b]
Lipid peroxidation (nM of MDA/mg protein)			
ND-CON	0.786 ± 0.082	1.021 ± 0.052	0.212 ± 0.022
STZ-CON	1.250 ± 0.180[a]	1.470 ± 0.185[a]	0.546 ± 0.052[a]
ND-L-NAME	0.790 ± 0.071	1.08 ± 0.063	0.257 ± 0.038
STZ-L-NAME	1.410 ± 0.189[a]	1.557 ± 0.740[a]	0.608 ± 0.085[a]
ND-L-NAME + PIO	0.790 ± 0.090	1.060 ± 0.088	0.190 ± 0.044
STZ-L-NAME + PIO	0.975 ± 0.130[b]	0.938 ± 0.144[b]	0.411 ± 0.038[b]

All the values are expressed as mean ± SEM ($n = 6–7$).
[a] Significantly different from ND-control group, $p < 0.05$.
[b] Significantly different from STZ-control group, $p < 0.05$.

4. Discussion

In the present study effect of pioglitazone treatment on L-NAME induced hypertension in STZ-diabetic rats was studied. Effect of pioglitazone treatment on blood pressure, vascular reactivity and antioxidant status was studied. Administration of STZ significantly increased blood glucose levels. Pioglitazone treatment had no effect on blood glucose levels as STZ-diabetic rats are insulin deficient and not insulin resistant. We have previously shown that pioglitazone administration does not have any effect on blood glucose levels in STZ model (Majithiya et al., 2005). Administration of L-NAME in diabetic and nondiabetic rats resulted in significant increase in blood pressure. This increase in blood pressure is due to blockade to nitric oxide synthatase. Blood pressure in diabetic rats treated with L-NAME was higher than in nondiabetic rats, this may be due to increased sympathetic tone (Kaufman et al., 1991) or increased rennin secretion (Woods et al., 1987) due to hyperglycemia in STZ-diabetic rats. This is in concurrence with other reports where in blood pressure was higher in L-

NAME diabetic rats (Fitzgerald and Brands, 2000). In the present study administration of pioglitazone had no significant effect on blood pressure in L-NAME induced hypertension in diabetic or nondiabetic rats. Moreover there was no significant change in Ach induced relaxation due to pioglitazone treatment in L-NAME treated rats. Various authors have reported blood pressure lowering effect of pioglitazone in fructose fed rats (Kotchen et al., 1997), insulin-resistant Rhesus monkey (Kemnitz et al., 1994), sucrose fed SHR (Uchida et al., 1997) and SHR (Grinsell et al., 2000). It has been reported that decrease in blood pressure due to pioglitazone is due to reduction in total peripheral resistance (Dubey et al., 1993) or direct dilation of vascular smooth muscles by blocking calcium channels (Zhang et al., 1994a,b; Buchanan et al., 1995). In the present study, blood pressure was not significantly changed in L-NAME diabetic–hypertensive rats due to pioglitazone treatment. This shows that reduction of blood pressure or increased Ach induced relaxation by pioglitazone treatment (Majithiya et al., 2005) is nitric oxide mediated. Earlier we have shown that treatment of pioglitazone reduces blood pressure, reduces oxidative stress and restores endothelial function in STZ-diabetic rats without having any significant effect on blood glucose levels.

Secondly, STZ administration caused significant increase in oxidative stress; various authors have also shown increase in oxidative stress in STZ model (Chang et al., 1993; Pieper, 1995). Oxidative stress is one of the major reasons of endothelial dysfunction in STZ model. There are various observations wherein some pharmacological free radical scavengers (Hattori et al., 1991; Pieper and Siebeneich, 1997a; Pieper, 1997), and some antioxidants (Karasu et al., 1997a,b) were able to improve the vascular dysfunction observed in diabetic vessels, thus demonstrating that oxidative stress is responsible for endothelial dysfunction. Further, the increased expression of superoxide dismutase isoforms reverses endothelial dysfunction in diabetic aorta (Zanetti et al., 2001). We have previously reported that pioglitazone reduces oxidative stress and this may also contribute as one of its reasons for reduction in blood pressure. In the present study pioglitazone administration significantly reduced oxidative stress. This is in concurrence with our earlier report and recent study in rabbits (Gumieniczek, 2003). In the present study although pioglitazone treatment reduced oxidative stress, it had no effect on blood pressure or on Ach induced relaxation in L-NAME treated rats. In case of diabetic rats increased oxidative stress leads to inactivation of nitric oxide, therefore administration of free radical scavengers or antioxidant therapy (Hattori et al., 1991; Pieper and Siebeneich, 1997a; Pieper, 1997; Karasu et al., 1997a,b) may reduce the breakdown of nitric oxide which leads to increase in bioavailability of nitric oxide and restore endothelial function. In the present study, though pioglitazone reduced oxidative stress in diabetic rats, there was no effect on blood pressure or Ach induced relaxation as there was complete absence of nitric oxide due to administration of L-NAME. Moreover various authors have previously shown that pioglitazone directly dilates blood vessels by blocking calcium channels (Zhang et al., 1994a,b; Buchanan et al., 1995). We have previously shown that direct vasorelaxant effect of pioglitazone exists only at a higher concentration but not at lower concentration. The present study supports our earlier observation (Majithiya et al., 2005) that reduction of blood pressure by pioglitazone at 10 mg/kg is nitric oxide mediated and not by calcium channel blocking effect of pioglitazone in STZ-diabetic rats. If pioglitazone reduces blood pressure by blocking calcium channel then in the present study pioglitazone treatment should reduce blood pressure in L-NAME hypertensive rats. But pioglitazone treatment produced no effect on blood pressure in L-NAME hypertensive diabetic or nondiabetic rats.

Hence from the present study it can be concluded that reduction in blood pressure in case of STZ-diabetic rats is nitric oxide mediated.

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ioglitazone, a PPARγ agonist, restores endothelial function in aorta of streptozotocin-induced diabetic rats

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Abstract

Objective: To study the effect of pioglitazone (a PPAR gamma agonist) treatment on blood pressure, endothelial function, and oxidative stress in streptozotocin (STZ)-induced diabetic rats.

Methods: Sprague–Dawley rats were randomized into control (n=32) and STZ-diabetic (n=32) groups. Rats were further randomized to receive pioglitazone (10 mg/kg) or placebo for 4 weeks, and the following protocols were carried out. Blood pressure, blood glucose level, and body weight were measured. Thoracic aorta was isolated and the dose–response curve of phenylephrine (PE) in the presence or absence of Nω-nitro-L-arginine-methyl ester (L-NAME) was recorded. The dose–response curve of acetylcholine (Ach) in the presence or absence of indomethacin, L-NAME, and methylene blue was recorded. Tone-related basal nitric oxide release experiments were carried out. Lipid peroxidation, superoxide dismutase, catalase, and reduced glutathione were estimated in liver, kidney, and aorta. Aortic nitrite levels were also measured. Further, in vitro effects of PE and Ach in the presence of pioglitazone (0.1 M–10 mM) were measured in aortic rings of nondiabetic and STZ-diabetic rats. Pioglitazone-induced relaxations were recorded in PE-contracted rings (with intact and denuded endothelium) in the presence of L-NAME and in KCl-contracted rings.

Results: Pioglitazone treatment reduced blood pressure without having any significant effect on blood glucose level and body weight of STZ-diabetic rats. Enhanced PE-induced contraction and impaired Ach-induced relaxations in STZ-diabetic rats were restored to normal by pioglitazone treatment. The presence of L-NAME but not indomethacin blocked Ach-induced relaxation in pioglitazone-treated STZ-diabetic rats. Basal nitric oxide release was significantly higher in pioglitazone-treated STZ-diabetic rats. Oxidative stress was significantly higher in STZ-diabetic rats and pioglitazone treatment significantly reduced it. High aortic nitrite levels of STZ-diabetic rats were significantly reduced by pioglitazone treatment. The presence of pioglitazone at higher concentrations (>10 μM), but not at lower concentrations, significantly changed the dose–response curve of PE or Ach. Pioglitazone relaxations at lower concentrations but not at higher concentrations were blocked by endothelium removal or by the presence of L-NAME.

Conclusion: Pioglitazone administration reduced oxidative stress, which prevented the breakdown of nitric oxide and increased nitric oxide levels, thereby restoring the endothelial function in aorta of STZ-diabetic rat. Hence, from the present study it can be concluded that pioglitazone administration in STZ-diabetic rats lowers blood pressure, protects against oxidative stress, and restores endothelial function.

1. Introduction

Cardiovascular disease is one of the leading causes of death in the western world and diabetes mellitus has been identified as a primary risk factor [1], due to which there is alteration in vascular responsiveness to several vasoconstrictors and vasodilators [2]. Most of the complications in diabetes are due to increased serum glucose and increased generation of oxygen-derived free radicals, which lead to endothelium dysfunction. It has been shown that vessels from diabetic animals exhibited abnormal endothelium dependent vascular relaxation to acetylcholine [3,4]. This endothelium-dependent vasodilation is reduced in diabetes largely due to excessive oxidative stress and decreased bioavailability of nitric oxide.

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Pioglitazone, a PPAR (peroxisome proliferators activated receptors) gamma agonist, improves insulin-mediated glucose uptake into skeletal muscle without increasing endogenous insulin secretion [5], and has been demonstrated to be effective in the treatment of non-insulin dependent diabetes mellitus with insulin resistance. It belongs to the thiazolidinedione class of drug and is an insulin sensitizer widely used in treatment of non-insulin-dependent diabetes mellitus. Pioglitazone lowers blood pressure and restores blunted endothelium-dependent vasodilatation in fructose-fed rats [6], insulin-resistant rheus monkey [7], SHR [8] and sucrose-fed SHR [9]. PPAR gamma agonists troglitazone, rosiglitazone, and pioglitazone all improve endothelial cell function in humans when measured by brachial artery responses to acetylcholine or when analyzed by small-vessel compliance [10]. Recently, a protective effect of pioglitazone against oxidative stress in liver and kidney of diabetic rabbits [11] has been reported. So far the effect of pioglitazone on blood pressure and endothelial function on aorta of streptozotocin-induced diabetic rats has not been studied. Hence, the purpose of the present study was to instigate the effect of pioglitazone treatment on blood pressure, endothelial function, and oxidative stress in streptozotocin-induced diabetic rats.

2. Materials and methods

2.1. Drugs

Pioglitazone hydrochloride was obtained as a gift sample from Alembic, Baroda. Streptozotocin, phenylephrine, acetylcholine, Nω-nitro-L-arginine-methyl ester (L-NAME), indomethacin, epinephrine, 1,1,3,3,-tetra ethoxy propane, superoxide dismutase, catalase and glutathione standard were obtained form SIGMA, St. Louis, MO, USA. All other chemicals and reagents used in the study were of analytical grade. Indomethacin was dissolved in 0.2 M Na_2CO_3. Stock solution of phenylephrine, acetylcholine, methylene blue, sodium nitroprusside, potassium chloride and L-NAME were prepared in double distilled water. For oral administration pioglitazone suspension was prepared in 0.5% sodium carboxy methyl cellulose and for in vitro studies, stock solution of pioglitazone was prepared in 0.1% DMSO [12]. The final concentration of DMSO in organ bath was less than 0.05% vol/vol. Stock solution of phenylephrine was stabilized with L-(+) ascorbic acid (1 μM), final concentration of ascorbic acid in organ bath was less than 0.1 pM.

2.2. Experimental protocol

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of M.S. University, Baroda and are in accordance with guidelines as per "Guide for the care and use of laboratory animals" published by NIH publication (No. 85-23 revised 1996) and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Male Sprague–Dawley rats (200±15 g) were housed in-group of 3 animals and maintained under standardized condition (12-h light/dark cycle, 24 °C) and provided free access to palleted CHAKKAN diet (Nav Maharashtra Oil Mills Pvt., Pune) and purified drinking water ad libitium. Diabetes was induced by single intravenous injection of streptozotocin (55 mg/kg, STZ) dissolved in normal saline. The control animals were injected with equal volume of vehicle. After 3 days following streptozotocin administration, blood was collected from tail vein and serum samples were analyzed for blood glucose (Enzymatic kits, GOD/POD method, SPAN diagnostics Pvt., India). Animals showing fasting blood glucose higher than 250 mg/dl were considered as diabetic rats and used for the study. Four weeks after induction of diabetes, blood pressure was measured by tail cuff method and rats with systolic blood pressure higher than 135 mmHg were selected, randomized into groups and used for the study. Age matched nondiabetic rats with systolic blood pressure less than 120 mmHg were randomized in to nondiabetic groups. Diabetic and nondiabetic rats were divided in to following groups: nondiabetic control (n=16, ND-CON), STZ-diabetic control (n=16, STZ-CON), nondiabetic group treated with pioglitazone (10 mg/kg/day) for 4 weeks (n=16, ND-PIO) and STZ-diabetic rats treated with pioglitazone (10 mg/kg/day) for 4 weeks (n=16, STZ-PIO). Respective control groups were orally administered with vehicle (1 ml/kg/day of 0.5% sodium carboxy methyl cellulose solution) for 4 weeks.

2.3. Blood pressure

Blood pressure was measured non invasively at the start of study and at weekly intervals by tail cuff method using LE 5002 storage pressure meter (LETICA scientific instruments, SPAIN) in all the above mentioned groups. For the blood pressure measurements animals were trained for at least 1 week until blood pressure was steadily recorded with minimal stress and restraint. The mean of 7–8 measurements of trained animals was recorded.

2.4. Preparation of aortic rings

The thoracic aorta of rats was isolated immediately after decapitation and carefully cleaned of fat and connective tissues. The aorta was cut into rings of 3 mm width. Extreme care was taken not to stretch or damage the luminal surface of the aorta to ensure the integrity of endothelium. In some rings endothelium was denuded by gently rubbing the aortic rings with forceps. Aortic rings were suspended between two 'S' shaped platinum loops in jacketed organ bath containing 20 ml krebs bicarbonate solution (pH 7.4) maintained at 37±0.5 °C and continuously aerated with 95% oxygen and 5% carbon dioxide. The composition of the Krebs solution (mM) was NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2,

$NaHCO_3$ 22.0, and glucose 11.0. The rings were connected to isometric force displacement transducer connected to Gemini pen recorder (UGO-BASILE, Italy). The rings were maintained under tension of 2 g and equilibrated for 90 min before initiating experimental protocol. During this period, the krebs solution was changed at every 15 min interval. After the equilibration period rings were maximally contracted with phenylephrine (PE, 1 μM) to test their contractile capacity, three recordings were carried out to find out a constant and reproducible contraction. The presence of functional endothelium was assessed by the ability of acetylcholine (Ach, 0.1 μM) to induce more than 60% of relaxation of rings precontracted submaximally with PE. Aortic rings were considered denuded when there was less than 10% relaxation to Ach.

2.5. Effect of PE and Ach on aortic rings obtained from control and pioglitazone treated rats

After 4 weeks of treatment aortic rings of ND-CON group, STZ-CON group, STZ-PIO group and ND-PIO group were isolated and mounted in organ bath as described above. Concentration–response curves to increasing concentrations of PE (1 nM–10 μM) were performed in rings with intact endothelium. Concentration–response curve of PE with the presence and absence of 100 μM of L-NAME was also recorded. Indomethacin (10 μM) was added to prevent the involvement of prostaglandins. Endothelium mediated relaxation was measured as a concentration–response curve to Ach (1 nM–10 μM) in rings precontracted with PE (80–90% of maximum response). Endothelium independent aortic relaxation to sodium nitroprusside (SNP, 0.001 nM–10 μM) was also measured in rings with denuded endothelium. Concentration dependent relaxation to Ach was recorded in precontracted rings 30 min after incubation with and in continued presence of 10 μM indomethacin (a non-selective cyclo-oxygenase inhibitor), 10 μM of methylene blue (a cGMP blocker) and 100 μM of L-NAME (a non-selective nitric oxide synthase inhibitor)+10 μM indomethacin. To investigate the effect of pioglitazone treatment on tone related basal nitric oxide release, aortic rings were submaximally (about 30–35%) contracted with PE (3 μM) and then response to addition to L-NAME (1–100 μM) was recorded as described by Hayashi et al. [13].

2.6. Measurement of superoxide dismutase, catalase, reduced glutathione and lipid peroxidation

After 4 weeks of treatment animals were sacrificed, liver, kidney and aorta were isolated and weighed [14]. The tissues were finely sliced and homogenized in chilled tris buffer at a concentration of 10% (w/v). The homogenates were centrifuged at 10,000×*g* at 0 °C for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for estimation of lipid peroxidation (MDA content), endogenous antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) and reduced glutathione (GSH)). Superoxide dismutase was determined by the method of Mishra and Fridovich [15]. Catalase was estimated by the method of Hugo Aebi as given by Hugo [16]. Reduced glutathione was determined by the method of Moron et al. [17]. Lipid peroxidation or malondialdehyde formation was estimated by the method of Slater and Sawyer [18].

2.7. Aortic nitrite levels

Nitric oxide (NO) easily breaks down with the presence of free radicals, hence aortic nitrite levels were measured as a level of NO inactivated due to superoxide radical (O_2^-). Nitrite was estimated colorimetrically with the Griess reagent [19] in aortic homogenate. Briefly equal volumes of aortic homogenate and Griess reagent (sulfanilamide 1% w/ v, naphthylethylenediamine dihydrochloride 0.1% w/v, and orthophosphoric acid 2.5% v/v) were mixed and incubated at room temperature for 10 min and the absorbance was determined at 540 nm wavelength. Nitrite was determined from the standard curve obtained using sodium nitrite as standard. The amount of nitrite formed was normalized to the protein content of the respective aorta.

2.8. Effect of the presence of pioglitazone on dose–response curves of PE, Ach and SNP in aortic rings of untreated animals

Aortic rings of untreated age matched nondiabetic (n=28) and STZ-diabetic (n=28) were mounted in organ bath as previously described. Concentration–response curve of PE and Ach were measured in rings (with intact endothelium), as described earlier, with the presence of DMSO (vehicle) or with pioglitazone (0.1 μM–10 mM). Relaxation to SNP (1 pM–100 μM) in endothelium denuded rings was also measured after pioglitazone incubation.

2.9. Effect of pioglitazone perse on PE and KCl contracted aortic rings of untreated animals

Concentration dependent relaxation of pioglitazone (10 nM–10 mM) in PE contracted rings with intact and denuded endothelium was recorded. Concentration dependent relax-

Table 1
Blood glucose level and body weight of rats before treatment (initial) and after 4 weeks of treatment (final) with pioglitazone

Groups	ND-CON	STZ-CON	ND-PIO	STZ-PIO
Blood glucose level (mg/dl)				
Initial	92±6.3	434±21.5[a]	96±9.4	452±16.7[a]
Final	89±8.2	448±17.4[a]	94±11.3[b]	458±23.8[a]
Body weight (g)				
Initial	221±14.8	219±23.6	217±8.5	221±21.3
Final	257±19.4	230±16.9	240±14.7	238±24.8

Values are expressed as mean±SEM.
[a] $p<0.05$, compared to ND-CON group.
[b] $p<0.05$, compared to STZ-CON group.

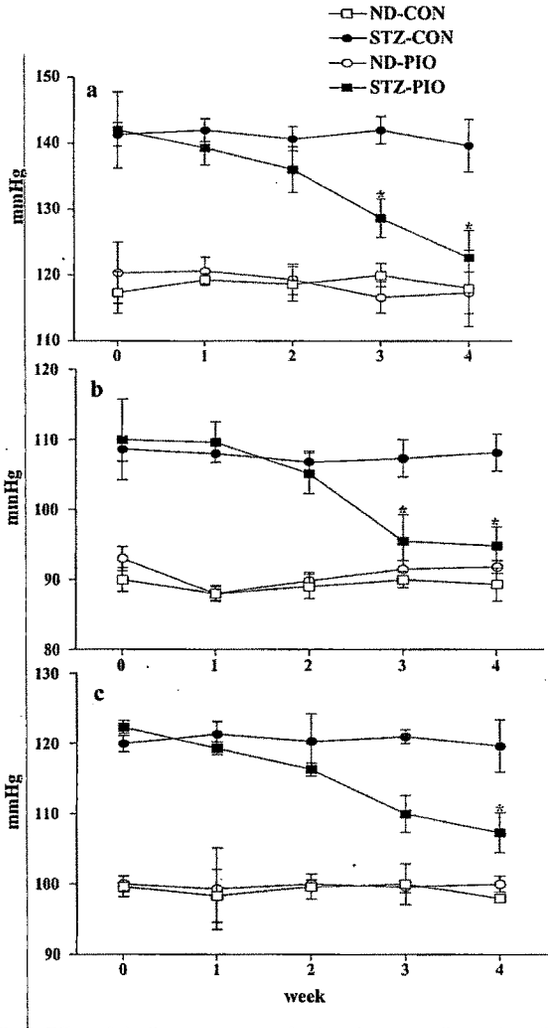

Fig. 1. Effect of pioglitazone treatment for 4 weeks on systolic (a), diastolic (b) and mean (c) blood pressure of ND-CON (-□-), STZ-CON (-●-), ND-PIO (-○-) and STZ-PIO (-■-) groups. Values are expressed as mean±SEM. * $P<0.05$, compared to STZ-CON group (n=5–8).

ation was recorded in precontracted (PE) rings (with intact endothelium) with the presence of 100 μM of L-NAME. Concentration dependent relaxation of pioglitazone (10 nM–10 mM) in endothelium denuded rings precontracted with 60 mM KCl was also recorded.

2.10. Statistical analysis

All the data are expressed as mean±SEM. Data were analyzed by ANOVA for repeated measurements followed by Bonferroni multiple comparison tests. Differences were considered to be statistically significant when $p<0.05$. The agonist pD_2 value ($-\log EC_{50}$) was calculated from concentration–response curve by non-linear regression analysis of the curve using computer based fitting program (Prism, Graphpad).

3. Results

3.1. Blood glucose and body weight

All streptozotocin injected animals developed diabetes. The changes in blood glucose levels are shown in Table 1. Blood glucose levels remained unchanged in nondiabetic animals (ND-CON and ND-PIO groups). There was significant ($p<0.05$) increase in blood glucose levels of streptozotocin injected animals. Pioglitazone treatment did not have any significant effect on blood glucose level of STZ-diabetic rats. Body weight of nondiabetic rats and STZ-diabetic rats showed a moderate increase. Pioglitazone treatment had no significant effect on the bodyweight of STZ-diabetic rat (Table 1).

3.2. Blood pressure

There was a significant ($p<0.05$) increase in systolic (142±11.41, mmHg), diastolic (104.5±10.165, mmHg) and mean blood pressure (117±10.58, mmHg) in STZ-CON group as compared to ND-CON (Fig. 1). Pioglitazone (10 mg/kg/day) treatment for 4 weeks significantly reduced systolic (124±9.23, mmHg), diastolic (94.5±9.2, mmHg) and mean blood pressure (107±9.21, mmHg) of STZ-PIO group as compared to STZ-CON group (Fig. 1). There was no significant change in systolic, diastolic or mean blood pressure of ND-PIO group treated with pioglitazone.

3.3. Contractile response to PE on aorta obtained from control and pioglitazone treated rats with the presence and absence of L-NAME

Cumulative addition of PE (1 nM–10 μM) resulted in concentration dependent contraction of aorta in all the groups (Fig. 2). There was a significant ($p<0.05$) increase in

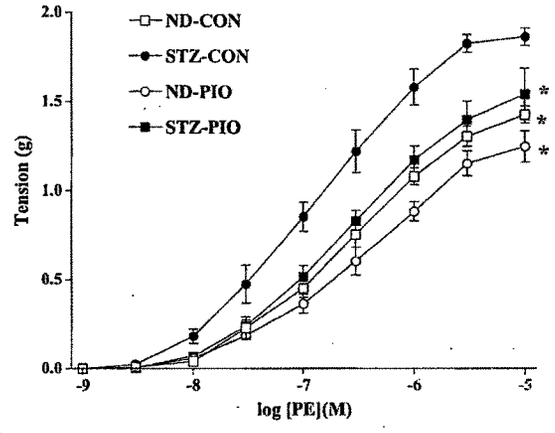

Fig. 2. Concentration response curve of phenylephrine on aortic rings obtained from ND-CON (-□-), STZ-CON (-●-), ND-PIO (-○-) and STZ-PIO (-■-) group. Values are expressed as mean±SEM. * $P<0.05$, compared to STZ-CON group. (n=5–8).

Table 2
Maximal response (E_{max}) and pD_2 (−Log EC_{50}) values of phenylephrine in presence and absence of L-NAME

Groups	Phenylephrine		Phenylephrine+L-NAME	
	pD_2	E_{max} (g)	pD_2	E_{max} (g)
ND-CON	6.69±0.026	1.418±0.14	6.89±0.044	1.84±0.042*
STZ-CON	6.96±0.046[a]	1.847±0.17[a]	6.89±0.056	1.85±0.048
ND-PIO	6.47±0.028	1.259±0.095	6.86±0.050	1.75±0.064*
STZ-PIO	6.62±0.038[b]	1.519±0.12[b]	6.84±0.042	1.80±0.037*

Values are expressed as mean±SEM.
[a] $p<0.05$, compared to ND-CON group.
[b] $p<0.05$, compared to STZ-CON group.
* $p<0.05$ when compared in same group in absence of L-NAME. (n=5–8).

maximal response (E_{max}) of PE in aorta obtained from STZ-CON group as compared to ND-CON group (Fig. 2). pD_2 value of PE in aorta obtained from STZ-CON group was significantly ($p<0.05$) higher as compared to ND-CON (Table 2). Contractile response of PE in aorta obtained from STZ-PIO group was attenuated as compared to STZ-CON group. Maximal response (E_{max}) and pD_2 value of PE in aorta obtained from STZ-PIO group was significantly ($P<0.05$) reduced as compared to STZ-CON group (Table 2). Maximal response (E_{max}) of PE in aortic rings of ND-CON, ND-PIO and STZ-PIO groups were significantly ($p<0.05$) increased due to the presence of L-NAME. pD_2 value of PE in aortic rings of ND-CON, ND-PIO and STZ-PIO groups were significantly ($p<0.05$) changed due to the presence of L-NAME (Table 2, Fig. 3). There was no significant change in maximal response (E_{max}) or pD_2 value of PE in aortic rings of STZ-CON group due to the presence of L-NAME (Table 2, Fig. 3).

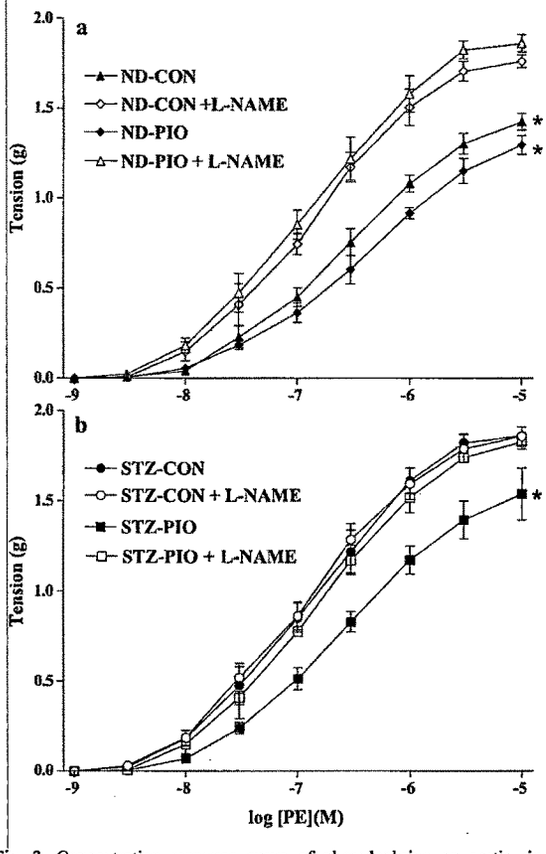

Fig. 3. Concentration response curve of phenylephrine on aortic rings obtained from (a) ND-CON (-▲-), ND-PIO (-◆-) and (b) STZ-CON (-●-), STZ-PIO (-■-) group in presence (light legends) and absence of 100 μM of L-NAME (dark legends). Values are expressed as mean±SEM. * $p<0.05$, compared to presence of L-NAME.

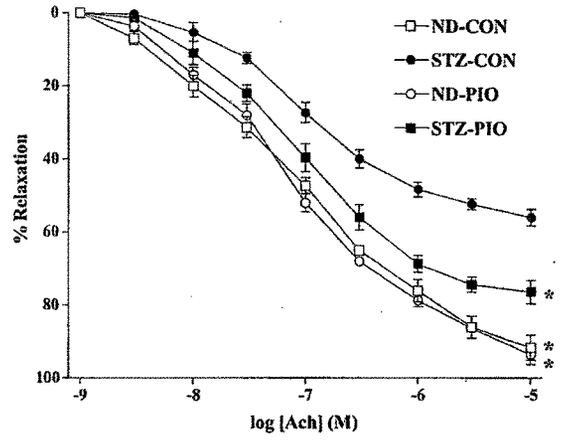

Fig. 4. Concentration dependent relaxation of acetylcholine on aortic rings obtained from ND-CON (-□-), STZ-CON (-●-), ND-PIO (-○-) and STZ-PIO (-■-) groups on aortic rings (with intact endothelium) precontracted with PE. Tension is expressed as % relaxation on initial contraction with PE. Values are expressed as mean±SEM. * $P<0.05$, compared to STZ-CON group. (n=5–8).

3.4. Relaxation response to Ach and SNP on aorta obtained from control and pioglitazone treated rats

Addition of Ach to all aortic rings with intact endothelium resulted in concentration dependent relaxation of rings that were precontracted with PE. Ach induced relaxation in aorta obtained from STZ-CON group was significantly ($p<0.05$) lower as compared to ND-CON group (Fig. 4). pD_2 value of Ach in STZ-CON group was significantly ($p<0.05$) lower as compared to ND-CON

Table 3
Maximal response (E_{max}) and pD_2 (−Log EC_{50}) values of acetylcholine

Groups	Acetylcholine	
	pD_2	E_{max} (% relaxation)
ND-CON	7.20±0.061	90.88±2.95
STZ-CON	6.96±0.065[a]	55.45±6.28[a]
ND-PIO	7.21±0.042	93.93±4.45
STZ-PIO	7.09±0.038[b]	75.63±2.73[b]

Values are expressed as mean±SEM.
[a] $p<0.05$, compared to ND-CON group. (n=5–8).
[b] $p<0.05$, compared to STZ-CON group.

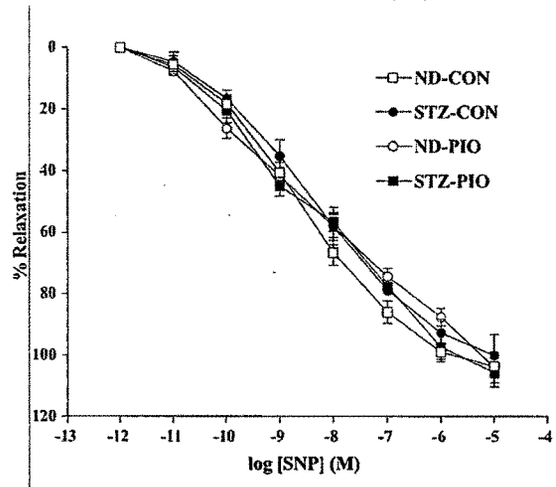

Fig. 5. Concentration dependent relaxation of sodium nitroprusside on endothelium denuded aortic rings obtained from ND-CON (-□-), STZ-CON (-●-), ND-PIO (-O-) and STZ-PIO (-■-) group. Tension is expressed as percentage relaxation of initial response to PE. Values are expressed as mean±SEM. (n=5–8).

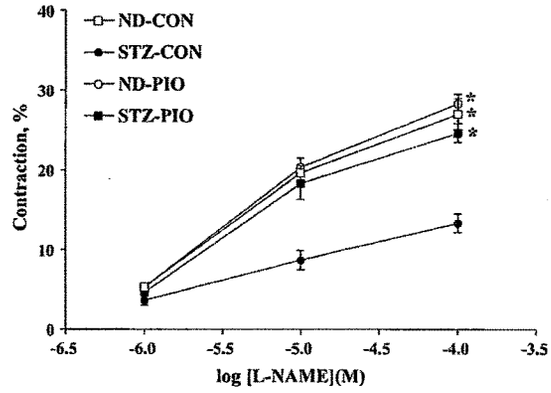

Fig. 7. Percent contraction of endothelium intact aortic rings to different concentration of L-NAME. The aortic rings obtained from ND-CON (-□-), STZ-CON (-●-), ND-PIO (-O-) and STZ-PIO (-■-) group were moderately contracted with phenylephrine before obtaining cumulative responses to L-NAME. Values are expressed as mean±SEM. * P<0.05, compared to STZ-CON.

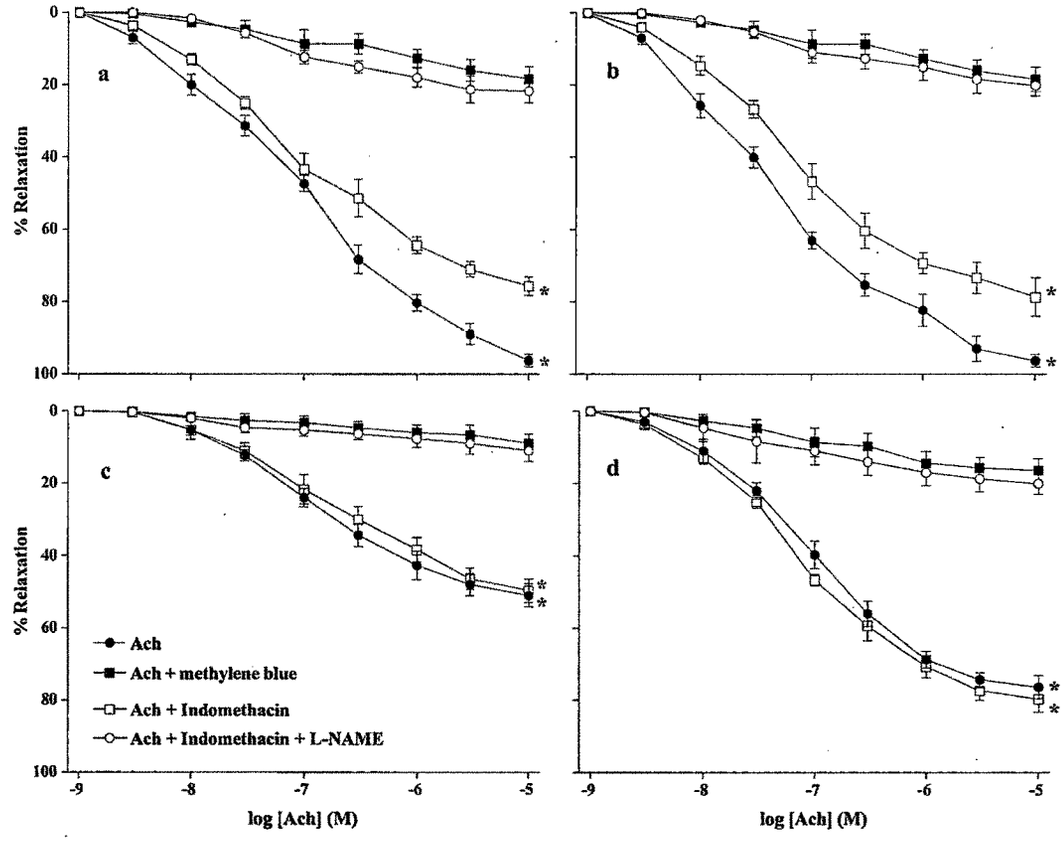

Fig. 6. Concentration dependent relaxation of acetylcholine alone (-●-) and in the presence of 10 μM of indomethacin (-□-), 100 μM of L-NAME+10 μM of indomethacin (-O-) and 10 μM methylene blue(-■-) in aortic rings (with intact endothelium) obtained from (a) ND-CON, (b) ND-PIO, (c) STZ-CON and (d) STZ-PIO group. Tension is expressed as percentage relaxation of initial response to PE. Values are expressed as mean±SEM. * P<0.05, compared to Ach+methylene blue and Ach+indomethacin+L-NAME. (n=5–8).

Table 4
Effect of pioglitazone treatment on superoxide dismutase, catalase, reduced glutathione and lipid peroxidation levels in liver, kidney and aorta

Groups	Liver	Kidney	Aorta
Superoxide dismutase (Unit/mg protein)			
ND-CON	7.53±0.134	9.23±0.048	5.591±0.108
STZ-CON	5.28±0.048[a]	5.17±0.195[a]	4.49±0.087[a]
ND-PIO	7.67±0.196	9.41±0.075	5.72±0.067
STZ-PIO	6.21±0.214[b]	6.53±0.153[b]	4.89±0.154[b]
Catalase (μM of H_2O_2 consumed/(min mg protein)			
ND-CON	11.26±0.27	11.43±0.59	5.78±0.054
STZ-CON	6.93±0.611[a]	6.21±0.73[a]	3.64±0.176[a]
ND-PIO	11.78±0.435	10.94±0.65	5.59±0.082
STZ-PIO	8.20±0.872[b]	7.54±0.39[b]	4.56±0.293[b]
Reduced Glutathione (μg of GSH/mg protein)			
ND-CON	9.86±0.072	11.21±0.043	2.61±0.181
STZ-CON	3.65±0.019[a]	3.42±0.324[a]	0.79±0.047[a]
ND-PIO	9.74±0.121	10.82±0.153	2.42±0.079
STZ-PIO	5.04±0.248[b]	5.30±0.367[b]	1.15±0.265[b]
Lipid Peroxidation (nM of MDA/mg protein)			
ND-CON	0.786±0.062	1.031±0.042	0.214±0.017
STZ-CON	1.240±0.169[a]	1.482±0.181[a]	0.538±0.052[a]
ND-PIO	0.795±0.093	1.070±0.068	0.195±0.064
STZ-PIO	0.978±0.138[b]	0.949±0.175[b]	0.407±0.039[b]

Values are expressed as mean±SEM.
[a] $p<0.001$, compared to ND-CON group ($n=6$–7).
[b] $p<0.01$ compared to STZ-CON group.

group (Table 3). Pioglitazone treatment significantly ($p<0.05$) increased Ach induced relaxation in aorta obtained from STZ-PIO group as compared to STZ-CON (Fig. 4). pD_2 value of Ach in STZ-PIO group was significantly ($p<0.05$) increased as compared to STZ-CON group (Table 3). Addition of SNP completely relaxed aortic rings of all the groups. There was no significant change in SNP induced relaxation on endothelium-denuded rings in any of the groups (Fig. 5).

3.5. Effects of indomethacin, L-NAME and methylene blue on Ach induced endothelium dependent relaxation on aorta obtained from control and pioglitazone treated rats

Ach completely relaxed precontracted aortic rings in ND-CON and ND-PIO groups and relaxation was completely blocked due to the presence of L-NAME or methylene blue (Fig. 6a, b). The presence of indomethacin decreased the relaxation of Ach in ND-CON and ND-PIO group. Ach induced relaxation which was impaired in STZ-CON group, was significantly increased in STZ-PIO group. Relaxation in STZ-CON and STZ-PIO was unaltered due to the presence of indomethacin, while it was completely blocked due to the presence of L-NAME or methylene blue (Fig. 6c, d).

3.6. Basal nitric oxide release

Addition of L-NAME to aortic preparation caused increase in contraction in all the groups. Contraction was significantly higher in STZ-PIO group aortic rings as compared to STZ-CON group (Fig. 7).

3.7. Superoxide dismutase, catalase, reduced glutathione and lipid peroxidation

Oxidative stress was significantly ($p<0.001$) increased in liver, kidney and aorta of STZ-CON group as compared to ND-CON group (Table 4). SOD, CAT and GSH were significantly decreased while lipid peroxidation was significantly increased in STZ-CON group. Pioglitazone treatment significantly ($p<0.01$) increased levels of endogenous antioxidants (SOD, CAT and GSH) in liver, kidney and aorta as compared to STZ-CON (Table 4). Moreover lipid peroxidation was significantly ($p<0.01$) decreased in liver, kidney and aorta of STZ-PIO group as compared to STZ-CON (Table 4). There was no significant change in SOD, CAT, GSH and lipid peroxidation on ND-PIO group as compared to ND-CON.

3.8. Aortic nitrite levels

Aortic nitrite levels of various groups are shown in Fig. 8. Aortic nitrite levels were significantly ($p<0.05$) higher in STZ-CON group as compared to ND-CON group. Pioglitazone treatment significantly ($p<0.05$) reduced aortic nitrite content of STZ-PIO group as compared to STZ-CON group. There was no significant change in aortic nitrite levels of ND-PIO group as compared to ND-CON group (Fig. 8).

3.9. Contractile effect of PE with the presence of pioglitazone on aorta obtained from untreated nondiabetic and STZ diabetic rats

The presence of pioglitazone (10 nM–10 mM) showed a similar pattern on contractile effect of PE in untreated ND and STZ-diabetic rats. Maximal response (E_{max}) and pD_2 value of ND and STZ-diabetic rats are shown in Table 5. Maximal response (E_{max}) was significantly

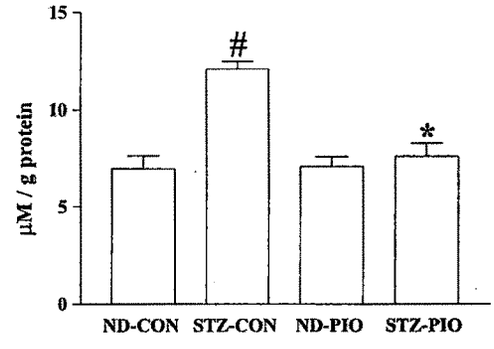

Fig. 8. Aortic nitrite levels determined from ND-CON, STZ-CON, ND-PIO and STZ-PIO groups. Values are expressed as mean±SEM. * $P<0.05$, compared to STZ-CON, #$P<0.05$, compared to ND-CON.

Table 5
Maximal response (E_{max}) and pD_2 (−Log EC_{50}) values of phenylephrine and acetylcholine on aortic rings of untreated age-matched non-diabetic and STZ-diabetic rats in presence of various concentration of pioglitazone

Pioglitazone	Non diabetic				STZ-diabetic			
	Phenylephrine		Acetylcholine		Phenylephrine		Acetylcholine	
	pD_2	E(max) g	pD_2	E(max) %relaxation	pD_2	E(max) g	pD_2	E(max) %relaxation
Vehicle (DMSO)	6.80±0.061	1.61±0.34	7.42±0.054	96.8±4.27	6.94±0.066	1.78±0.459	6.91±0.049	55.4±5.45
0.1 μM	6.76±0.030	1.58±0.37	7.43±0.041	98.2±3.82	6.86±0.062	1.77±0.045	7.02±0.049	56.8±7.83
1 μM	6.67±0.033	1.61±0.25	7.36±0.089	97.8±6.30	6.74±0.061	1.72±0.044	7.05±0.053	60.7±6.78
10 μM	6.55±0.025*	1.56±0.29*	7.48±0.071	99.4±3.76	6.79±0.061*	1.61±0.041*	7.11±0.063*	80.6±5.91*
100 μM	6.53±0.048*	1.48±0.32*	7.71±0.062*	102.1±2.4*	6.67±0.076*	1.57±0.053*	7.27±0.588*	86.3±8.93*
1 mM	6.27±0.032*	1.53±0.16*	7.80±0.059*	103.4±2.89*	6.62±0.063*	1.53±0.043*	7.43±0.663*	89.7±6.99*
10 mM	6.25±0.034*	1.52±0.18*	7.96±0.056*	105.3±2.49*	6.59±0.086*	1.49±0.058*	7.41±0.717*	96.3±8.10*

Values are expressed as mean±SEM.
* $p<0.05$ compared to vehicle. (n=5–8).

higher in case of STZ-diabetic rats as compared to ND rats (Table 5). Contractile response of PE in high concentration (greater than 10 μM) of pioglitazone was significantly ($p<0.05$) decreased as compared to vehicle (Fig. 9). Contractile response of PE was unaltered with the presence of low concentration (less than 10 μM) of pioglitazone , while the maximal response (E_{max}) to PE was decreased as the concentration of pioglitazone exposed to the aortic rings was increased (Table 5). The presence of pioglitazone at higher concentrations significantly ($p<0.05$) decreased the pD_2 values of PE induced contractile response (Table 5). The presence of pioglitazone caused a concentration dependent rightward shift in PE response (Fig. 9).

3.10. Relaxation response to Ach and SNP with the presence of pioglitazone on aorta obtained from untreated nondiabetic and STZ diabetic rats

Ach completely relaxed aorta obtained from untreated ND rats while relaxation in aorta obtained from STZ-diabetic rats was impaired with the presence of vehicle (E_{max},% relaxation: 55.4±1.45%). Relaxation response to Ach in low concentration of pioglitazone (less than 10 μM), was unaltered in ND or STZ-diabetic rats (Fig. 10). There was significant ($P<0.05$) increase in Ach induced relaxation in aortic rings in higher concentration of pioglitazone (greater than 10 μM) in aortic rings of ND and STZ-diabetic rats (Fig. 10). Percent relaxation produced by Ach was significantly ($p<0.05$) enhanced with the presence of pioglitazone (greater than 10 μM) as compared to vehicle in ND as well as STZ-diabetic rats (Table 5). The presence of higher concentration of pioglitazone caused significant ($p<0.05$) change in pD_2 values of relaxation response to Ach (Table 5). Concentration–response curve of Ach in aorta of untreated ND rats was shifted towards left with the presence of pioglitazone (Fig. 10). SNP induced relaxation in case of ND rats (data not shown) and STZ-diabetic rats were similar and there was no significant effect of SNP induced relaxation due to the presence of pioglitazone (10 nM–10 mM) as compared to vehicle (Fig. 11).

3.11. Relaxation response to pioglitazone on precontracted aorta obtained from untreated nondiabetic and STZ diabetic rats

Addition of pioglitazone (10 nM–10 mM) in PE contracted rings (with intact endothelium) produced concentration dependent relaxation in ND and STZ-diabetic rats (Fig. 12). The relaxation was blocked due to the presence of L-NAME at lower concentration (less than 10 μM), but not at higher concentration. Pioglitazone did not produce relaxation at lower concentration (less than 10 μM) in endothelium denuded aortic rings of ND and STZ-diabetic rats, whereas at concentration higher than 10 μM pioglitazone produced relaxation (Fig. 12). Addition of pioglitazone at higher concentrations (greater than 10 μM) produced relaxation in endothelium denuded aortic rings that were contracted by 60 mM K^+, but did not relax at lower concentration (Fig. 12).

4. Discussion

Administration of STZ caused significant elevation in blood glucose level of diabetic rats and treatment with pioglitazone had no significant effect on blood glucose concentration in both nondiabetic and diabetic rats. The reason for this could be that the streptozotocin-induced diabetic animals were not insulin-resistant but insulin-deficient. Glitazone reduce plasma glucose levels by increasing peripheral insulin sensitivity (GLUT 4) and by additional effects on liver and skeletal muscle. Hence in STZ-diabetic animals (insulin-deficient), there was no significant effect of blood glucose levels. Similarly, pioglitazone did not affect the body weight, which moderately increased in nondiabetic as well as in diabetic rats. The blood pressure of 8-week STZ-diabetic rats was significantly higher as compared to nondiabetic control.

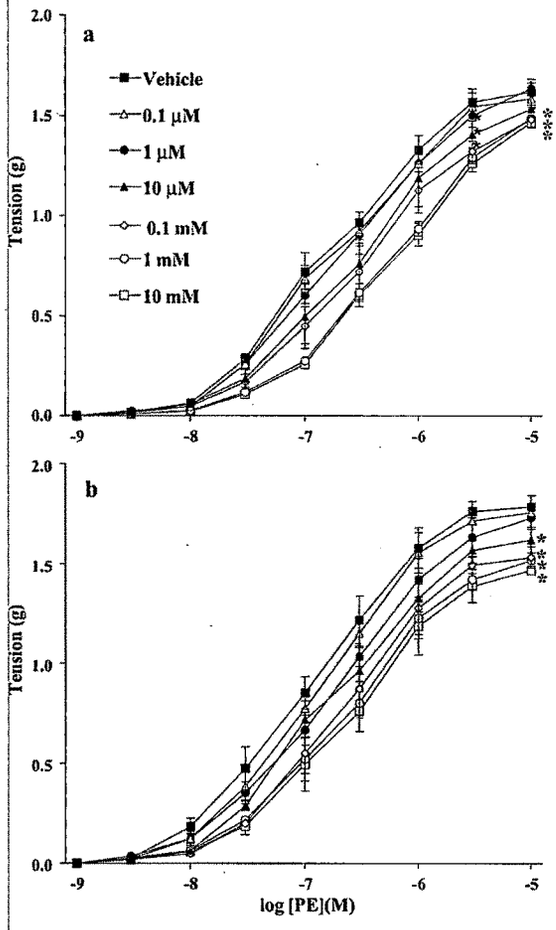

Fig. 9. Concentration response curve of phenylephrine on aortic rings obtained from untreated age matched non-diabetic rats (a) and STZ diabetic rats (b) in presence of varying concentration of pioglitazone ((-■-) vehicle-DMSO and 0.1 μM (-△-), 1 μM (-●-), 10 μM (-▲-), 0.1mM (-◇-), 1 mM (-○-) and 10 mM (-□-) of pioglitazone). Values are expressed as mean±SEM. * $P<0.05$, compared to vehicle. (n=5–8).

The results from the isolated aortic studies demonstrated that aortas from 8-week STZ-diabetic rats (STZ-CON) are more responsive to PE, while the relaxation response to Ach was significantly decreased than those from ND-CON. Similar results showing the increased vascular responsiveness to PE and decreased Ach induced relaxation in STZ-diabetic rats have been reported in previous studies [20–22]. Moreover the levels of endogenous antioxidants (SOD, CAT and GSH) were significantly reduced and lipid peroxidation significantly increased in STZ-CON group showing increased oxidative stress. Similar results showing increased oxidative stress (increased lipid peroxidation and reduced SOD, CAT and GSH) have been reported [23] in previous studies in STZ model.

Enhanced contractility could be due to deficient endothelial activity [24,25], enhancement of oxidative stress due to excessive production of oxygen-free radicals and decreased antioxidant defense systems [26,27]. It is a well-known fact that endothelium-dependent relaxation response to agonists such as Ach is impaired in diabetic rat aorta [3,4]. There are two possible mechanisms for reductions in Ach-induced relaxation, first nitric oxide (NO)-dependent vasodilatation, i.e. a decrease in NO release (or production) from the endothelium and a decreased reactivity of vascular smooth muscle to NO in diabetic animals. Another possible mechanism of reduced responses to Ach in diabetic animals is that oxidative degradation and inactivation of NO may be increased in vessels of such rats. Several studies have indicated the increased production of superoxide anions in vessels of diabetic animals. Further it is suggested that this active form of oxygen can inactivate NO to attenuate NO-dependent vasodilatory response [28] in the diabetic rabbit aorta [29]. It has also been reported that oxidative stress increases diacylglycerol-protein kinase activity in aorta of hyperglycemic rats [30] and leads to

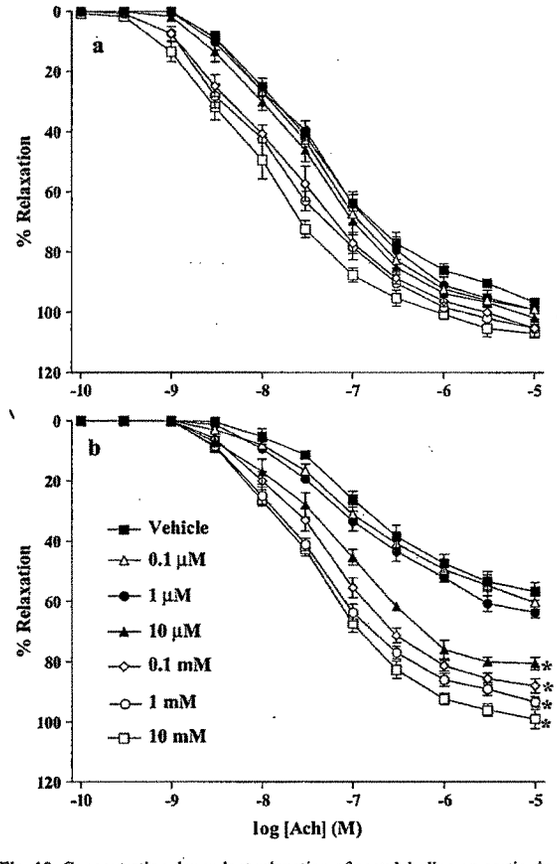

Fig. 10. Concentration dependent relaxation of acetylcholine on aortic rings (with intact endothelium) of untreated age matched non-diabetic rats (a) and STZ diabetic rats (b), precontracted with PE and in presence of varying concentration of pioglitazone ((-■-) vehicle-DMSO and 0.1 μM (-△-), 1 μM (-●-), 10 μM (-▲-), 0.1 mM (-◇-), 1 mM (-○-) and 10 mM (-□-) of pioglitazone). Tension is expressed as percentage relaxation of initial response to PE. Values are expressed as mean±SEM. * $P<0.05$, compared to vehicle. (n=5–8).

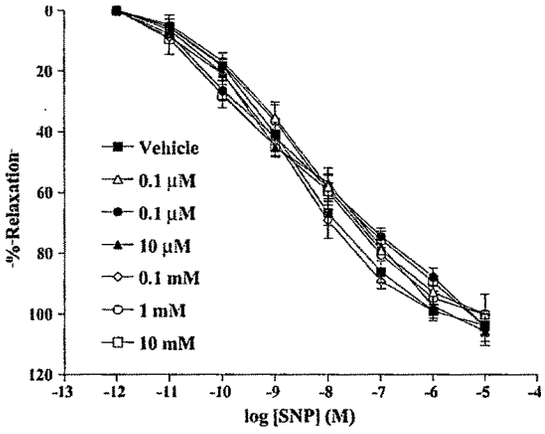

Fig. 11. Concentration dependent relaxation of sodium nitroprusside on endothelium denuded aortic rings of untreated age matched STZ diabetic rats precontracted with PE and in presence of varying concentration of pioglitazone ((-■-) vehicle-DMSO and 0.1 μM (-△-), 1 μM (-●-), 10 μM (-▲-), 0.1mM (-◇-), 1 mM (-○-) and 10 mM (-□-) of pioglitazone). Tension is expressed as percentage relaxation of initial response to PE. Values are expressed as mean±SEM. (n=5–6).

impaired endothelium-dependent relaxation in STZ-diabetic rat aorta. STZ-diabetic rats showed increased oxidative stress along with enhanced vascular contractility and decreased Ach induced relaxation. Therefore, the oxidative stress in diabetic animals might be responsible for increased contractility together with deficient endothelial function [25,31].

Administration of pioglitazone for 4 weeks restored the elevated blood pressure, reduced the enhanced contractility to PE and Ach induced relaxation was restored. In pioglitazone treated STZ-diabetic rats there was an increase in Ach induced relaxation which may be due to involvement of NO pathway since the relaxation was blocked with the presence of L-NAME and not with the presence of indomethacin. Moreover relaxation to Ach was also blocked with the presence of cGMP blocker methylene blue suggesting role of cGMP in elevated relaxation to Ach in STZ-diabetic aorta. Further tone related basal nitric oxide studies showed that pioglitazone treatment significantly increased the basal nitric oxide release in aortas of STZ-diabetic rats.

Various authors have shown that pioglitazone directly dilates blood vessels by blocking calcium channels [32,33]. In vitro studies on aorta of nondiabetic and STZ-diabetic rats were carried out to investigate whether the blood pressure lowering effect is due to direct effect of pioglitazone by blocking calcium channels. PE induced contraction and Ach induced relaxation studies with various concentrations of pioglitazone showed that the presence of low concentration of pioglitazone did not have any effect on the PE induced contraction or Ach induced relaxation. But the presence of higher concentration (greater than 10 μM) of pioglitazone caused significant changes in dose–response curves of PE and Ach in STZ-diabetic and nondiabetic aortas, showing direct effect of pioglitazone exists at concentration higher than 10 μM. Moreover as concentration increases from 10 μM to 100 mM direct vasodilator effect of pioglitazone increases. This was further evidenced as pioglitazone induced relaxation in rings with intact endothelium was blocked with the presence of L-NAME at lower concentrations but not at higher concentrations. Further relaxation due to pioglitazone in endothelium denuded rings depolarized by 60 mM K^+ also supported this fact. Pharmacokinetic study in male rats have shown that maximum plasma concentration (Cmax) of pioglitazone after oral administration of 10 mg/kg is 35 μM [34]. As maximum plasma concentration after oral administration of 10 mg/kg of pioglitazone is 35 μM and in vitro data shows direct effect of pioglitazone at concentration greater than 10 μM, hence in the present study some direct effects of pioglitazone dose exist. But blood pressure lowering effect cannot be completely attributed to direct effect of pioglitazone on calcium

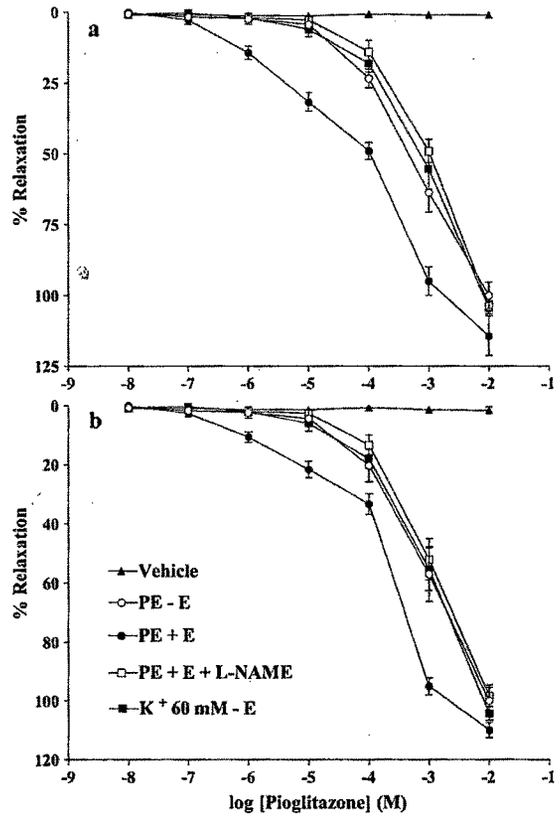

Fig. 12. Concentration dependent relaxation of pioglitazone on untreated age matched non diabetic rats (a) and STZ diabetic rats (b), precontracted with (-●-) PE with intact endothelium (+E), (-○-) PE with denuded endothelium (-E), (-□-) PE with intact endothelium (+E) in presence of 100 μM L-NAME, (-■-) K^+60 mM with denuded endothelium (-E) and (-▲-) vehicle. Values are expressed as mean±SEM. (n=5–8).

channels as blood pressure was not lowered after the first week of treatment, significant blood pressure lowering effect was observed only after the third week of pioglitazone treatment. This may be due to the fact that though there may be direct effect of pioglitazone at week 1, but as STZ induced endothelial dysfunction is prominent and not restored, as a result blood pressure is not lowered after 1 week of pioglitazone treatment. Blood pressure lowering effect is observed only after 3 weeks when endothelial function is restored. Hence restored endothelial function together with direct effect of pioglitazone on calcium channels may be the reason for the decrease in blood pressure after pioglitazone treatment.

The reduction in oxidative stress may also be one of the reasons of the decrease in blood pressure coupled with restored endothelium function of STZ-diabetic rats treated with pioglitazone. Nitric oxide is rapidly inactivated by O_2^- and it has been reported that an enhanced formation of O_2^- radical may be involved in the accelerated breakdown of nitric oxide [35,36]. Moreover it has been shown that rapid destruction of nitric oxide occurs in streptozotocin induced diabetic rats [37]. The protective effect of pioglitazone against oxidative stress may prevent the breakdown of nitric oxide, which may improve vascular function. Similar observations were reported that pioglitazone reduces oxidative stress and increases NO bioavailability in coronary arterioles of mice [38]. Dobrian et al. [39] have reported that pioglitazone administration prevents hypertension and reduces oxidative stress in diet induced obesity. Similarly Kanie et al. [40] have reported bezafibrate, a PPAR alpha agonist improves endothelium-dependent relaxation by increasing expressions of the mRNAs for PPAR alpha and PPAR gamma. This may lead to a decrease in the expression of prepro ET-1, and the consequent decrease in plasma ET-1 may cause a decline in the expression of NAD(P)H oxidase, thereby resulting in a decrease in superoxide anion and a normalization of the endothelial dysfunction. It is also reported that PPAR gamma agonists reduce blood pressure in patients with type 2 diabetes and hypertension [41] and obese patients without diabetes [42,43].

Hence the restored endothelial function could be attributed to the protective effect of pioglitazone against oxidative stress and blood pressure lowering effect could be attributed to combined effect of restored endothelial function and direct effect of pioglitazone. Hence from the present study it can be concluded that pioglitazone administration in STZ-diabetic rats lowers blood pressure, protects against oxidative stress and restores endothelial function.

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