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Anti-ischemic Effects of Nimesulide, a Cyclooxygenase-2 Inhibitor on the Ischemic Model of Rabbit Induced by Isoproterenol

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The objective was to devise an animal model of myocardial infarction (MI) against which cardioprotective drugs might be tested. We describe the effects of nimesulide, a COX experience with development and validation of such a model. The rabbit was chosen in preference to rodents because its heart and cardiac circulation more closely resemble those of human. Thus, the cardiovascular system of anaesthetized male rabbits, 1 to 1.5 kg (n=11), was stressed by a single bolus intravenous injection of isoprenaline (ISP), 65 mg/kg. The effects of the injection were followed for sixteen days and were evaluated in four ways: 1) measurements of creatinine kinase isozyme and troponin-I (TPI) in serum 2) Electrocardiographic (ECG) changes (ST elevation and Q wave development) 3) Cardiac histopathology observed in tissue sections of the isolated of the heart. The histopathological analysis showed that rabbit heart on 2nd day after ISP injection showed changes of coagulation necrosis. Day 4 total coagulation with the loss of nuclear and striation associated with heavy interstitial infiltrate of neutrophils was found. Day 8 after infarction showed collagen deposition with capillary channels in between the remaining islands of myocytes in the infarcted area. On the 16th day scarring was complete. Coronary perfusion rates (CPR) and heart rate (HR) of the infarcted and nimesulide (a COX-2 inhibitor) treated rabbits displayed significant improvement (n=11) on each corresponding day after infarction as compared to the infarcted and saline treated rabbits ($P<0.05$). All four indices revealed similarities with effects commonly associated with MI in humans.

Key words: Isoprenaline (ISP), Troponin I (TPI), Creatine Phosphokinase (CPK), Electrocardiography (ECG), Histopathology, Coronary perfusion rates (CPR)

INTRODUCTION

The development of cyclooxygenase-2 (COX-2) inhibitors as anti-inflammatory agents without gastric toxicity is based on the fact that COX-1 predominates in stomach yielding cytoprotective prostaglandin E2 (PGE-2), while COX-2 is induced in inflammation, giving rise to pain, swelling and discomfort (Mukerjee, 2002). The fact that COX-2 is an inducible enzyme particularly associated with inflammation led to the development of selective COX-2 inhibitors that offer comparable efficacy and fewer unwanted side effects attributable to COX-1 inhibition, gastric ulceration in particular (Bombardier *et al.*, 2000; Silverstein *et al.*, 2000). Gastrointestinal safety of selective COX-2 inhibitors, however, may come at the cost of increased cardiovascular events, as suggested by the VIGOR trial (Mukerjee *et al.*, 2001).

The available clinical data with COX-2 inhibitors pertaining to cardiovascular endpoints was summarized recently (Mukerjee *et al.*, 2001). This data suggests the risk of cardiovascular events associated with the use of COX-2 inhibitor when used for arthritis. Studies performed by Duffy *et al.* (1999) have defined the relative roles of vasodilator prostaglandins in patients with atherosclerosis. In these studies, vasodilator prostaglandins were demonstrated to mediate metabolic vasodilatation and flow-mediated vasodilatation in response to rapid cardiac pacing in patients with atherosclerosis (Duffy *et al.*, 1999). At the other extreme is the possibility that COX-2 antagonists may serve to improve vascular health and retard atherosclerosis.

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Studies have shown that COX-2 is widely expressed in atherosclerotic lesions and may colocalize with inducible nitric oxide synthase and nitrotyrosine within macrophages (Baker *et al.,* 1999). Some studies show that short-term treatment with the COX-2 specific inhibitor rofecoxib and the nonselective COX antagonist naproxen does not impair endothelium-dependent or -independent vascular function in healthy volunteers. However, the role of COX-2 during experimentally induced ischemia is still unclear. In the present investigation we studied the anti-ischemic effects of nimesulide-a COX-2 inhibitor, in rabbits subjected to isoproterenol (ISP), an important â agonist. In this model the acute phases of myocardial necrosis and repair mimicked those, which occurred in humans (Saeed *et al.,* 1998). We have used nimesulide as a COX-2 inhibitor in our study.

METHODS AND MATERIALS

Male rabbits 1 to 1.5 kg were housed for at least 7 days before an experiment in the Animal Unit of HEJ Research Institute of Chemistry, University of Karachi. They were fed a standard rabbit chow with water freely available ad libitum.

Rabbits were divided into three groups of ten animals each. One group consisted of normal rabbits, second group consisted of infarcted rabbits treated with saline and the third group consisted of infarcted rabbits that were treated with nimesulide (a COX-2 inhibitor). All infarcted rabbits received nimesulide 25 mg/kg or saline 0.5 mL every day, up to and including the day of sacrifice. Experiments were performed on day 2, 4, 8 and 16 post-infarction.

On day two of experiment after taking the electrocardiographic (ECG), the blood samples for enzyme assays were obtained from the marginal ear vein. The rabbits were sacrificed and hearts removed and used for histopathological studies. After Langendorff study, the hearts were subjected to histological studies.

Myocardial infarction (MI)

In the present study, MI was induced by a single, parenteral dose of beta-adrenergic agonist, ISP (65 mg/kg). For many years the main technique for producing MI in animals was to manually occlude with a silk suture the anterior descending coronary artery of the anesthetized rat. A recent method calls for a single large parenteral dose of the β-adrenergic agonist, isoproterenol. (Saeed *et al.,* 1998). The advantages of isoproterenol-induced infarction, which occurs as a result of intense ionotropic and chronotropic actions of isoproterenol compared to physical occlusion of coronary artery, are;

(1) Production of experimental MI with β-adrenergic agonist is by comparison, less invasive and accomplished without the complicating factor of general anesthesia.
(2) No foreign body (suture) remains on or in the heart.
(3) Reperfusion is possible after isoproterenol since there is no permanent overt occlusion.
(4) Reported survival rates with isoproterenol are better than after vessel occlusion.

Enzyme levels

1. Troponin I (TPI) was analyzed using IMMULITE Turbo Troponin I Analyzer (Adams *et al.,* 1993). Samples were loaded on bar-coded sample cup and then placed onto a load platform. We then loaded the bar-coded test units. Pressed "GO" and the test units were conveyed to the analyzer for bar-coded identification and then moved on to a main incubation carosel. The pipetter added sample and reagent and the test units were incubated at 37°C at 16 minutes. The test units were shuttled to the spin/wash station, where bound and free labels were separated. Substrate was then added and the test units were then transferred to the luminometer chain. 10 minutes incubation at 37°C began which caused the signal to reach maximum limits. The photon count was measured with a photo amplifier tube.
2. Creatine phosphokinase (CPK) reagent was used to measure the CPK activity by an enzymatic rate method (Rosalki, 1967). The SYNTRON CX System automatically proportions the appropriate sample and reagent volumes into the cuvette. The ration used is one sample to 20 parts reagent. The system monitors the change in absorbance at 340 nanometers. This change in absorbance is directly proportional to the activity of CPK in the sample and is used by the SYNTRON CX system to calculate and express creatine phosphokinase activity. Similarly blood for CPK was also drawn at 0, 4, 10, 16 and 28 h after isoproterenol injection from the marginal ear vein of the rabbit.

Electrocardiography (ECG)

ECG was obtained using modified Einthoven system. One lead each placed over the right and left rib cage vs. an indifferent lead on the left lower leg. Lead I to record potentials across the thorax, positive on the left and negative on the right. Lead II from the right thorax to the left leg has a negative to positive polarity.

Histopathology

Rabbits were killed; chest cavities opened and hearts were removed. After carrying out the Langendorff experiment, hearts were fixed in 10% buffered formalin. After proper fixation, the hearts were measured, bisected and entirely submitted into two cassettes. Tissue processing

was done by routine methodology. After processing, the tissues were embedded in paraffin using the histocentre from Shenden. 3-5 μm thick sections were cut by Microtom AS 325 from Shenden and stained with hematoxylin and eosin (H & E). Selected sections were stained with trichrome and interpreted under an Olympus BX 50 microscope.

Langendorff isolated heart preparation

Coronary perfusion rate (CPR) was measured using Langendorff isolated heart preparation (Langendorff, 1985). Rabbits were killed by cervical dislocation and heart removed rapidly and placed in oxygenated Krebs Hansliet Solution (KHS). The pericardium was removed; a segment of aorta left attached. Residual blood was removed by massaging the heart in KHS. The heart was then transferred to the Langendorff apparatus (Bioscience Kent, UK.) where it was tied to a glass cannula. KHS warmed at 37°C and bubbled with 95% oxygen and 5% carbon dioxide was passed from an elevated reservoir at a constant pressure of 60 cm of water. This pressure causes the aortic valve to close, but permits the perfusate to enter the coronary circulation. When perfusion established, the heart began to beat to beat spontaneously. We let the heart stabilize till the contractions became rhythmic (usually 30 minutes).

Statistical analysis was done using one-way ANOVA followed by Bonferroni test for selected pair of groups. Differences were considered significant when probability (p) was <0.05.

RESULTS

The results of the enzymes levels are shown in Fig. 1A and 1B. To confirm that MI had occurred by ISP, blood for troponin I was drawn at 0, 4, 10, 16 and 28 h after ISP injection from the marginal ear vein of the rabbit. The results with TPI and CPK obtained show a continuous increase in the serum level of the enzyme which is suggestive of MI. This confirmed that MI had taken place in the rabbits.

The results of ECG also demonstrate (Fig. 2) that MI had occurred. ECG taken after ISP injection showed ST wave elevation and development of Q wave was also found. These findings further confirmed that infarction had taken place in the rabbit heart.

Photomicrographs of the rabbit heart muscle on the 2nd day after ISP injection shows changes of coagulation necrosis (Fig. 3, day 2). Ischaemic myocytes with pyknosis of nuclei, shrunken eiosinophilic cytoplasm and marginal contraction band necrosis is seen. On the 4th day, total coagulation necrosis with the loss of nuclei and striation was observed (Fig. 3, day 4). We also found heavy interstitial infiltrate of neutrophils. On the day eight, collagen deposition with capillary channels in-between the remaining islands of myocytes in the infarcted area and prominent fibrovascular reaction in margins are seen (Fig. 3, day 8). On the 16th day scarring was complete. We noted prominent scarring with scattered remaining darkly stained myocytes (Fig. 3, day 16). All these changes are similar to those take place in the human heart after an acute MI.

Coronary perfusion rate

We observed a steady decrease in CPR in infracted rabbits as compared to the normal rabbits ($p<0.05$) on the day 2 after infarction (Fig. 4A). On day 4 of post-infarction, there was further drop in CPR which reached its maximum decline on day 8 of post-infarction (Fig. 4B and 4C). Following day 8 of post-infarction, CPR started to recover and values returned close to those of day 2, although still away from the values of the normal rabbits (Fig. 4D).

Infarcted rabbits were divided into two groups. One group was treated with saline on the day of infarction and

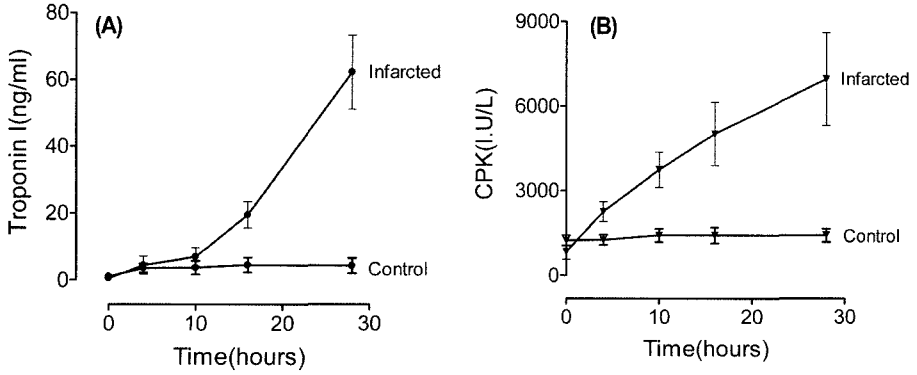

Fig. 1. (A) Troponin I levels in the blood at different time points after isoproterenol injection and (B) CPK levels in the blood at different time points after isoproterenol injection

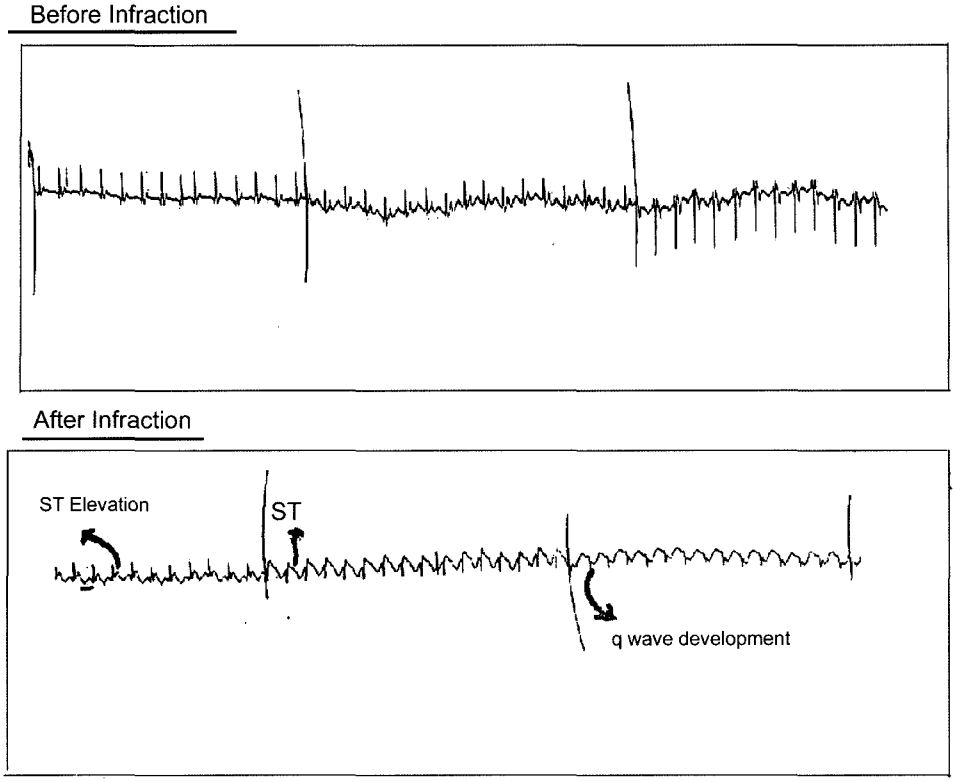

Fig. 2. ECG Tracings after Isoproterenol Injection

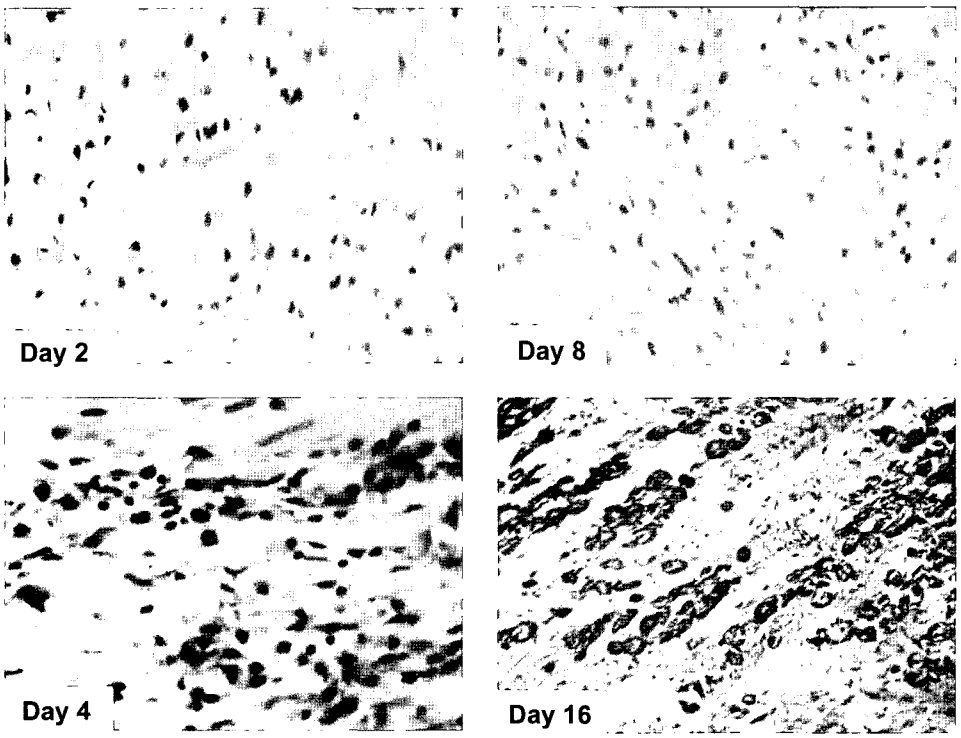

Fig. 3. Photomicrographs of the rabbit heart muscle (left ventricle), (day 2) after 24-28 hours of infarction. Ischemic myocytes show changes of coagulation necrosis. H & E. Magnification 20X (day 4) after 72-76 hours of infarction. Note florid predominantly acute inflammatory cell infiltrate around necrotic myocytes (arrow). H & E. Magnification 10X on day 8 after infarction. Note collagen deposition with capillary channels in-between the remaining islands of myocytes in the infarcted area (arrow). H & E. Magnification 10X (day 16) Photomicrograph of the rabbit heart muscle (left ventricle) on day 16 after infarction. Note prominent scarring with scattered remaining darkly stained (arrow myocytes. Trichrome Magnification 20X.

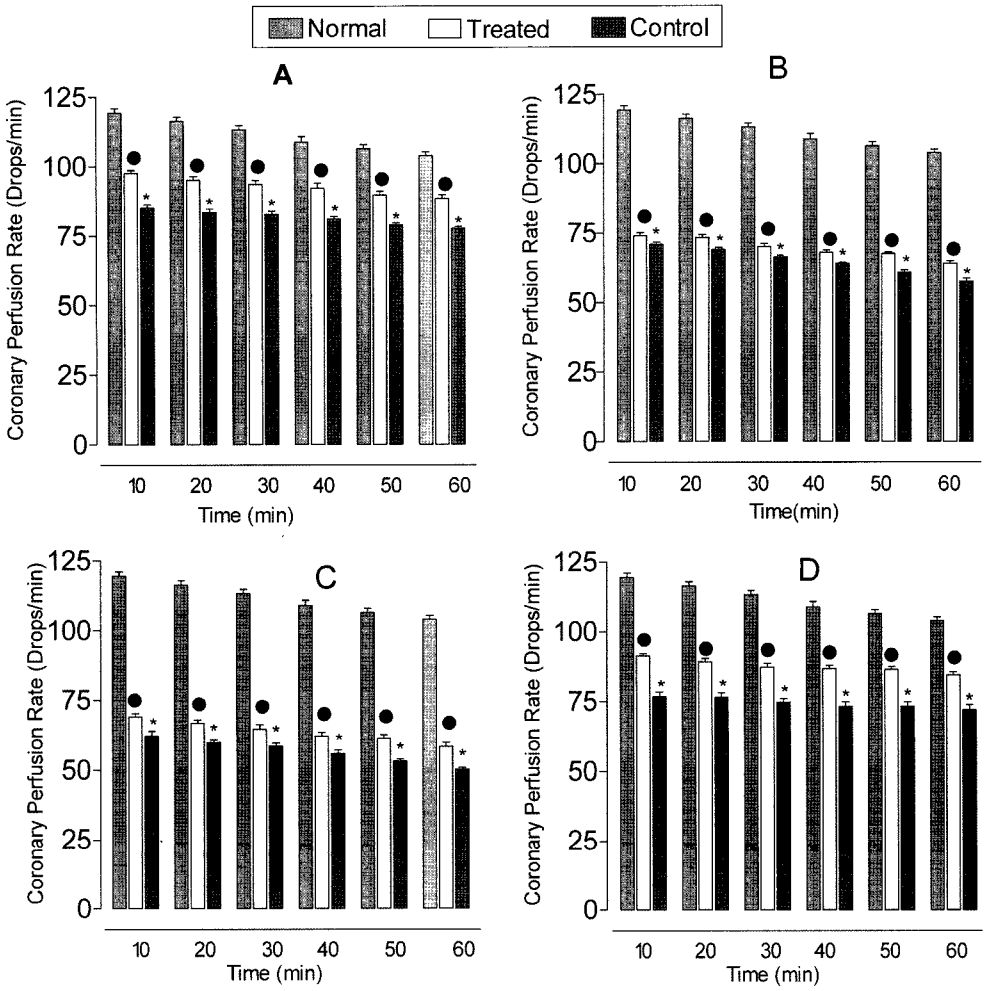

Fig. 4. Coronary perfusion rates of infarcted plus nimesulide treated rabbits vs. infarcted plus saline treated rabbits (controls) on day 2 (A), on day 4 (B), on day 8(C) and on day 16 (D) after infarction. N=6, *p<0.05 for control rabbits compared to nimesulide-treated group. • p<0.05 for nimesulide-treated rabbits compared to normal, non-infarcted group. Rabbits in the infarcted plus nimesulide treated group showed significant improvement in the coronary perfusion rate as compared to the rabbits in the infarcted and saline treated group on each corresponding day after infarction.

this treatment continued up to the day of sacrifice. Similarly the other group was treated with nimesulide on the day of infarction and this treatment continued up to the day of sacrifice. Third group was non-infarcted, normal rabbits.

On day 2 post-infarction, CPR on the two groups of the rabbits were compared. Rabbits in the nimesulide treated group showed significant improvement in CPR ($p<0.05$) was compared to the saline treated rabbits. On day 4 post-infarction, rabbits in the nimesulide treated infracted group again displayed significant improvement in CPR ($p<0.05$) as compared to the saline treated rabbits. It means that nimesulide treatment made recovery in CPR towards normal, although still very low then normal ($p<0.05$). On day 8 of post-infarction, there was significant improvement in CPR of nimesulide treated infarcted rabbits ($p<0.05$). On day 16 of post-infarction CPR of the two groups of infarcted rabbits were compared. Nimesulide treated infarcted rabbits displayed significant improvement ($p<0.05$) than the saline treated infarcted rabbits.

On day 16 of post-infarction, values of CPR of both groups of infarcted rabbits better than the infarcted rabbits of day 4 and day8 post-infarction. On day 16 post-infarction rabbits treated with nimesulide showed maximum recovery. These values were very close to those of nimesulide treated rabbits at day 2 post-infarction. These values of CPR, however, were still significantly lower than the normal ($p<0.05$), nevertheless, showing that on day 16 post -infarction, rabbits in the nimesulide treated group made significant recovery.

DISCUSSION

In the present study, we have developed a model for experimental MI. The main features of this model follow changes seen in humans after MI. For example, the

Table I. Coronary perfusion rates (CPR) of normal, infarcted plus saline, infarcted plus nimesulide treated rabbits on day 2, 4, 8 and 16 after infarction

Time (Min)	Coronary Perfusion Rate (Drops/Min)								
	Normal	Day 2		Day 4		Day 8		Day 16	
		Sal (Infarcted)	Nim (Infarcted)	Sal (Infarcted)	Nim (Infarcted)	Sal (Infarcted)	Nim (Infarcted)	Sal (Infarcted)	Nim (Infarcted)
10	117±6	85±7	98±7	71±6	74±5	74±5	76±5	75±4	86±3
20	114±7	84±6	95±5	69±5	73±4	73±5	76±6	74±3	84±4
30	112±7	83±7	93±5	66±6	70±5	70±4	75±7	73±5	83±3
40	107±8	81±8	92±7	64±4	68±4	68±3	73±6	72±4	83±4
50	105±9	79±6	90±4	61±4	67±4	67±5	73±6	72±4	82±4
60	102±7	78±5	88±8	57±5	64±3	64±6	72±7	70±3	80±4

changes seen in the levels of TPI and CPK from myocytes. These findings further corroborated the induction of MI by ISP. The application of this model for evaluating drugs which might interfere with clotting and heart attacks, in particular nimesulide, a COX-2 inhibitor which also known to reduce oxidative stress and exhibit antioxidant properties.

The other class in particular aspirin, non-steroidal inflammatory drugs (NSAIDs), which is known to inhibit platelet aggregation mediated by COX. Its has clinical efficacy in ischaemic heart disease (Saeed *et al.,* 2001). It beneficial effects are probably due to its antithrombogenic actions as an inhibitor of platelet aggregation. However, a direct cardioprotective effect of aspirin on ischaemic myocardium has not been demonstrated. Studies have shown that aspirin did not affect myocardial infarct size after canine coronary artery infarction (Bonow *et al.,* 1981). It has been reported (Mobert *et al.,* 1998) that aspirin caused a 15-fold increase in guinea pig heart and concluded that nonspecific COX inhibition leads to myocardial oxygen deprivation. Thus, selective COX-2 inhibition seemed to have clear therapeutic advantage.

COX-2 is the isoform responsible for the enhanced production of prostaglandins that mediate inflammation and is the target enzyme for the anti-inflammatory activity of NSAIDs (Lecomte *et al.,* 1994). Induction of COX-2 in ischaemic myocardium is thought to increase the production of proinflammatory prostanoids and contribute significantly to the ischemic inflammation (Saito *et al.,* 2000). Furthermore, COX-2 expression is induced by proinflammatory mediators, particularly by cytokines and reactive oxygen species (Belton *et al.,* 2000), and its expression has been found in animal models of atherosclerosis as well as in human atherosclerotic tissues (Baker *et al.,* 1999; Schonbeck *et al.,* 1999). It has proposed that atherosclerosis is a process with inflammatory features (Koening, 2001) and selective COX-2 inhibitors may potentially have antiatherogenic effects by the virtue of inhibiting inflammation (Mukerjee *et al.,* 2002).

Improvement in the coronary perfusion rate found in the present studies (Fig. 4) strongly suggests that coronary vasodilatation occurs through endothelial dependant NO formation. An increasing body of evidence suggests that oxidative stress accounts in large parts for endothelial dysfunction (Cai *et al.,* 2000). There is evidence that COX-2 may be a source of oxygen radicals itself (O'Banion, 1999) and therefore, inhibition of this enzyme activity by nimesulide may reduce oxidative stress. The link between increased oxidative stress and reduced bioavailability of NO has been well established (Cai *et al.,* 2000). Furthermore, endothelial dysfunction in patients with coronary artery disease is beneficially reversed by anti-oxidative agents, such as vitamin C (Hetzer *et al.,* 1996). In fact selective COX-2 inhibition holds the potential to beneficially impact outcome in patients with cardiovascular disease (Chenevard *et al.,* 2003).

More recently, it has been demonstrated that selective COX-2 inhibition improves endothelial-dependent vasodilatation and reduces low-grade chronic inflammation and oxidative stress in coronary artery disease (Chenevard *et al.,* 2003).

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REFERENCES

Adams, J. E. 3rd., Bodor, G. S., Davila-Roman, V.G., Delmez, J. A., Apple, F. S., Ladenson, J. H., and Jaffe, A. S., Cardiac troponin I: a marker with high specificity for cardiac injury. *Circulation,* 88, 101-106 (1993).

Baker, C. S., Hall, R. J., Evans, T. J., Pomerance, A., Maclouf, J., Creminon, C., Yacoub, M. H., and Polak, J.M., Cyclooxygenase-2 is widely expressed in atherosclerotic lesions affecting native and transplanted human coronary arteries

and colocalized with inducible nitric oxide synthase and nitrotyrosine particularly in macrophages. *Arterioscler. Thromb. Vasc. Biol.*, 19, 646-655 (1999).

Belton, O., Byrne, D., Kearney, D., Leahy, A., and Fitzgerald, D. J., Cyclooxygenase-1 and -2 dependent prostacyclin formation in patients with atherosclerosis. *Circulation*, 102, 840-845 (2000).

Bombardier, C., Laine, L., Reicin, A., Shapiro, D., Burgos-Vargas, R., Davis, B., Day, R., Ferraz, M. B., Hawkey, C. J., Hochberg, M. C., Kvien, T. K., and Schnitzer, T. J., Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis: VIGOR Study Group. *N. Engl. J. Med.*, 243, 1520-1528 (2000).

Bonow, R. O., Lipson, L. C., Shaheen, F. H., Capurro, N. L., Inser, J. M., Roberst, W.C., Goldstein, R. E., and Epstein, S.E., Lack of effect of aspirin on myocardial infarct size in dogs. *Am. J. Cardiol.*, 47, 258-264 (1981).

Cai, H. and Harrison, D. G., Endothelial dysfunction in cardiovascular disease: the role of oxidant stress. *Cirs. Res.*, 87, 840-844 (2000).

Chenevard, R., Hurlimann, D., Bechir, M., Enseleit, F., Spieker, L., Hermann, M., Riesen, W., Gay, S., Gay, R. E., Neidhart, M., Michel, B., Luscher, T. F., Noll, G., and Ruschitzka, F., Selective COX-2 inhibition improves endothelial function in coronary artery disease. *Circulation*, 107, 405-409 (2003).

Duffy, S. J., Castle, S. F., Harper, R. W. *et al.*, Contribution of vasodilator prostanoids and nitric oxide to resting flow, metabolic vasodilatation, and flow-mediated dilation in human coronary circulation. *Circulation,* 100, 1951-1957 (1999).

Heitzer, T., Just, H., and Munzel, T., Antioxidant vitamin C improves and endothelial dysfunction in chronic smokers. *Circulation,* 94, 6-9 (1996).

Koenig, W., Inflammation and coronary heart disease: an overview. *Caridol. Rev.*, 9, 31-35 (2001).

Langendorff. *Pfluners Arch. Ges. Physiol.*, 190, 280 (1985).

Lecomte, M., Laneuville, O., Ji, C., DeWitt, D. L., and Smith, W. L., Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J. Biol. Chem.*, 269, 13207-13215 (1994).

Mobert, J. and Becker, B. F., Cyclooxygenase inhibition aggravates ischaemia-reperfusion injury in the perfused guinea pig heart: involvement of isoprostanes. *J. Am. Coll. Cardiol.*, 31, 1687-1694 (1998).

Mukerjee, D., Nissen, S. E., and Topol, E. J., Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA.* 286, 954-959 (2001).

Mukerjee, D., Selective Cyclooxygenase-2 (COX-2) inhibitors and potential risk of cardiovascular events. *Biochem. Pharmacol.,* 63, 817-21 (2002).

O'Banion M., Cyclooxygenase-2: Molecular biology, pharmacology and neurobiology. *Critc. Rev. Neurobiol.,* 13, 45-82 (1999).

Rosalki, S. B., An improved procedure for serum creatine phosphokinase determination. *J. Lab. Clin. Med.,* 69, 696-705 (1967).

Saeed, S. A., Atiq, M., Virani, S., and Gilani, A. H., New vistas in the therapeutic uses of aspirin. *Res. Commun. Pharmacol. Toxicol.,* 6, 277-296 (2001).

Saeed, S. A., Gilani, A. H., Sultan, B. H., Karim, R. M., and Shah, B. H., Myocardial ischemia and infarction in isoprenaline-treated rabbits: Role of cyclooxygenases. *Bichem. Soc. Trans.*, 26, S342 (1998).

Saito, T., Rodger, I. W., Hu, F., Shennib, H., and Giaid, A., Inhibition of cyclooxygenase-2 improves cardiac function in myocardial infarction. *Biochem. Biphys. Res. Commun.*, 273, 772-775 (2000).

Schonbeck, U., Sukhova, G. K., Graber, P., Coulter, S., and Libby, P., Augmented expressoin of cyclooxygenase-2 in human atherosclerotic lesions. *Am. J. Pathol.*, 155, 1281-1291 (1999).

Silverstein, F. E., Faich, G., Goldstein, J. L., Simon, L. S., Pincus, T., Whelton, A., Makuch, R., Eisen, G., Agrawal, N. M., Stenson, W. F., Burr, A. M., Zhao, W. W., Kent, J. D., Lefkowith, J. B., Verburg, K. M., and Geis, G. S., Gastrointestinal toxicity with celecoxib vs nonsterodial anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS Study. A randomized control trial. *JAMA,* 284, 1247-1255 (2000).