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Augmented myocardial ischaemia by nicotine – Mechanisms and their possible significance

[1,2]Karsten Schrör, [1]Katja C. Zimmermann & [1]Roman Tannhäuser

[1]Institut für Pharmakologie, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany

1 To study the effect of nicotine on the severity of experimental myocardial ischaemia, Langendorff hearts of rabbits ($n=7-12$ per group) were subjected to 2 h of low-flow ischaemia followed by 1 h of reperfusion.

2 Infusion of nicotine (100 ng ml^{-1}) caused only minor changes in non-ischaemic conditions but a significant ($P<0.05$) increase in end-diastolic pressure (LVEDP), loss of creatine kinase (CK) and troponin (TnT) as well as increase in noradrenaline (NA) overflow in reperfused ischaemic hearts.

3 RT–PCR was done on total RNA for mRNA expression of the constitutive (COX-1) and inducible cyclooxygenase (COX-2). There was no COX-2 in non-ischaemic hearts but a significant expression in ischaemia ($n=5$) which was further increased by nicotine. These data were confirmed at the protein level by Western blotting and additionally shown that COX-1 remained unchanged.

4 There was a marked increase in prostacyclin (PGI_2) and a 2 fold increase in NA overflow which were both stimulated by nicotine.

5 The aggravating effects of nicotine on myocardial ischaemia (CK release) as well as the expression of COX-2 mRNA were prevented by pretreatment with the β-blocker pindolol (1 μM).

6 The data demonstrate marked deleterious actions of nicotine in reperfused ischaemic hearts. These actions are probably related to the increase in catecholamine overflow, are β-receptor-mediated and involve enhanced gene expression of COX-2.

Keywords: β-Blocker; catecholamines; COX-2 gene regulation; prostacyclin

Introduction

Cigarette smoking is an established risk factor for myocardial infarction and sudden cardiac death. Current estimates are a 2 fold increase in myocardial infarction and infarct-related death in smokers as compared to non-smokers in the United States (Heart and Stroke Facts, 1995) and Europe (Doll *et al*., 1994). Tobacco smoke contains a plethora of potentially toxic products, however, it is the nicotine which probably mediates the toxic effects of cigarette smoke in the cardiovascular system and determines the risk to suffer and eventually to die from vascular diseases (Bounameaux *et al*., 1988; Rose, 1996).

While there is no question that chronic nicotine intake via cigarette smoking increases the risk of suffering a myocardial infarction, several studies have also demonstrated that the outcome of infarcted patients who are current smokers might be better than in ex-smokers or non-smokers (Kelly *et al*., 1985; Robinson *et al*., 1988; Grines *et al*., 1995; Zahger *et al*., 1995). A recent analysis from patients of the GUSTO-1 trial (GUSTO-1 Investigators, 1995) has shown that smokers, suffering from myocardial infarction, had a substantially lower 30-day mortality rate than non-smokers (Barbash *et al*., 1993). There was also a more successful restoration of coronary blood flow after thrombolysis and a significantly lower rate of reocclusion in smokers (Barbash *et al*., 1993; De Chillou *et al*., 1996). It has even been suggested that smoking is independently associated with lower mortality (Molstad, 1991). There is currently no explanation for this 'smoking paradox', i.e. an increased risk of thromboembolic events in the cardiovascular system but a possibly improved outcome.

Nicotine acts *via* nicotinic receptors in the central and peripheral nervous system, eventually resulting in enhanced sympathetic and parasympathetic outflows (Löffelholz, 1979). Consequently, nicotine might affect vessel tone, platelet function and cardiac contractility *via* an increased noradrenaline release. Clinical studies have shown that cigarette smoking increases plasma noradrenaline and adrenaline levels and that this might be related to vascular toxicity (Cryer *et al*., 1976; Winniford *et al*., 1986). Intravenous nicotine has been found to increase infarct size in dogs (Masden & Flowers, 1980; Sridharan *et al*., 1985) and it is possible that an increased noradrenaline release was involved. Exposure to tobacco smoke for some weeks also causes a significant increase in infarct size in rats (Zhu *et al*., 1994). It is well known that marked noradrenaline overflow occurs in ischaemic hearts (Schömig *et al*., 1984). This catecholamine overflow during longer periods of ischaemia (i.e. >10 min) results in a redistribution of catecholamines in the ischaemic area of the ventricle (Holmgren *et al*., 1981; Schrör *et al*., 1981a; Muntz *et al*., 1984) and is initiated by intraneuronal release of catecholamines from their storage vesicles (Schömig, 1990). Thus, nicotine-induced catecholamine release might stimulate the ischaemia-related catecholamine overflow and increase the severity of myocardial ischaemia.

We have shown that ischaemia-related redistribution of cardiac catecholamines, i.e. the redistribution of noradrenaline from the perivascular adrenergic nerve terminals into extraneuronal tissue, can be reversed or prevented by prostacyclin (PGI_2) (Schrör *et al*., 1981a,b; 1982; Schrör & Funke, 1985). Indomethacin has the opposite effect and enhances both catecholamine overflow and myocardial injury (Schrör & Funke, 1985). This suggests that endogenous PGI_2 has some protective effect on ischaemic injury *via* reduction of catecholamine redistribution. Interestingly, several clinical studies have shown an increased generation of the cycloox-

[2]Author for correspondence.

ygenase (COX) products PGI_2, prostaglandin E_2 (PGE_2) and thromboxane in the circulation after smoking or nicotine administration (Toivanen *et al*., 1986; Nowak *et al*., 1987; Riutta *et al*., 1995). However, the opposite has also been found (Nadler *et al*., 1983; Lennon *et al*., 1988). Similar conflicting results were reported *in vitro*, even in the same system, such as isolated cultured human endothelial cells or isolated hearts (Wennmalm, 1978b; Bull *et al*., 1988; Boutheron-Falson & Blaes, 1990). The information from these reports is that smoking might somehow interfere with cyclooxygenase product formation and that nicotine is probably the mediator (Riutta *et al*., 1995). It should be noted that all of these investigations solely studied prostaglandin formation. There is no data on COX-protein expression or even COX-gene regulation. COX exists in two isoforms. The constitutive isoform (COX-1) and the inducible isoform (COX-2) (Otto & Smith, 1995). The inducible isoform is stimulated by inflammatory and immunogenic mediators and responsible for pathophysiological effects of prostaglandins while the constitutive isoform is mainly responsible for physiological effects of prostaglandins (Hla & Neilson, 1992; Wu, 1995). So far, the inducible COX-2 isoform has been found in a number of organs and tissues, including vascular smooth muscle and endothelial cells but not cardiac tissue of several species (Tippetts *et al*., 1988; Feng *et al*., 1993) including men (O'Neill & Ford-Hutchinson, 1993).

This study demonstrates that COX-2 becomes expressed in rabbit cardiac tissue after ischaemia and that this expression is enhanced by exposure to nicotine. Administration of nicotine to ischaemic hearts also results in a marked aggravation of myocardial ischaemic injury by nicotine, possibly subsequent to enhanced catecholamine release and overflow and can be largely prevented by β-receptor blockade.

Methods

Heart preparations

The experiments were performed in accordance with the guidelines of the American Heart Association on Research Animal use and approved by the German State Authority. Langendorff hearts were prepared from New Zealand White rabbits (1.6–2.2 kg) as previously described (Woditsch & Schrör, 1992). The hearts were perfused at a constant rate of 22 ml min^{-1} with Krebs-Henseleit-buffer (pH 7.4), containing 3 μM oxyhaemoglobin. The temperature of the heart was kept constant at 37.0 ± 0.1°C by a surrounding water-jacket. The perfusion buffer was equilibrated with 95% oxygen and 5% CO_2 by using a membrane oxygenator (Eschweiler, Kiel, Germany). This resulted in a pO_2 of >200 mm Hg in the coronary effluent under non-ischaemic conditions, indicating sufficient oxygenation of the hearts.

Functional measurements

The hearts were beating spontaneously. Left ventricular systolic (LVSP) and end-diastolic pressures (LVEDP) were measured by a Statham transducer connected to a balloon inserted into the cavity of the left ventricle. Coronary perfusion pressure (CPP) was measured by another Statham transducer in the aortic inflow tract. These parameters were monitored continuously and recorded on a Mac-Lab-Laboratory computer. Heart rate (HR) was computed from left ventricular pressure signals.

Determination of PGI_2

PGI_2 release was measured in aliquots of the coronary effluent in terms of the stable hydrolysis product 6-oxo-prostaglandin-$F_{1\alpha}$ ($PGF_{1\alpha}$) by radioimmunoassay (Schrör & Seidel, 1988).

Biochemical measurements in the coronary effluent

Creatine phosphokinase (CK)-specific activity was determined in the coronary effluent as described elsewhere (Woditsch & Schrör, 1992). Troponin T was determined with the ELISA one-step sandwich assay in another aliquot of the coronary effluent according to recommendations of the producer (Boehringer Mannheim, Germany).

RNA-extraction

At the end of the experiment, the hearts were removed and immediately frozen in liquid nitrogen. Total cellular RNA was prepared from left ventricles, using a modified Trizol-extraction technique. Briefly, the ventricles were homogenized three times for 30 s at 20,000 r.p.m in icecold Trizol-solution using an Ultraturrax homogenizer (Polytron PT10/35, diameter 12 mm; Kinematica Luzern, Switzerland). After phenol/chloroform extraction, RNA was precipitated with isopropanol at 0°C overnight. The final supernatant, containing purified RNA, was washed once with 75% ethanol and then resuspended in distilled water. The RNA was quantified by measuring the optical density at 260 nm (Spectrophotometry) and the degradation was checked by the appearance of the 18S and 28S RNA bands after agarose-formaldehyde gel electrophoresis and ethidium bromide staining.

Reverse transcription

A reverse transcription (RT) of RNA was performed in 20 μl of buffer containing 1 $\mu g\ \mu l^{-1}$ total RNA, 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 1 mM dNTP's, 2.5 U μl^{-1} RNasin, 2.5 μM random hexamers, and 10 U μl^{-1} reverse transcriptase (MMLV). The samples were incubated at 42°C for 15 s. Afterwards the enzyme was inactivated by heating to 99°C for 5 min.

Polymerase Chain Reaction (PCR) for determination of COX-2

A PCR reaction was performed, in which 80 μl of PCR master mix (3 mM $MgCl_2$ 50 mM KCl, 10 mM Tris; pH 8.3), 80 μmol of sense and antisense primers and 2.5 U of AmpliTaq DNA polymerase were added to 20 μl of reverse-transcriptase reaction mix at a final volume of 100 μl. This sense and antisense primers were designed to generate a 410-base pair product spanning the bases 1440 to 1920 of the rabbit cyclooxygenase-2 (COX-2). As an internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used which generated a 210 base pair product in every single PCR reaction. The thermal reaction profile consisted of denaturation at 95°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 2 min. The amplification was terminated at 40 cycles and the reaction mix was cooled down to 4°C.

After PCR, 3 μl of loading buffer (Sigma, Gel Loading Solution) was added to 10 μl aliquots containing the PCR products. The PCR products were separated by electrophoresis on 1.5% ethidium bromide-gels. The 420 bp PCR product, corresponding to COX-2 was quantified in terms of the 210 bp product, corresponding to GAPDH by scanning of the gels

and measurement of light intensity by a videosystem (Biometra, Göttingen, Germany).

Western blotting

Left ventricular tissue was homogenized in lysis buffer, containing 0.15 M NaCl, 0.1 M Tris-buffered saline (pH 7.4), 50 mM diethyldithiopyrocarbonate, 1% (v/v^{-1})Tween-20, and 10 mM phenylmethylsulfonylfluoride, PMSF. Protein concentration was measured with a Bio-Rad-kit, following the procedures described by Bradford (1976). Samples of the lysate (50 μg protein each) were applied on a 8% (w/v^{-1}) SDS polyacrylamide-gel, electrophoresed and transferred to nitrocellulose membranes. The membranes were saturated with a solution of 5% fat-free milk in phosphate-buffered saline, 0.05% (v/v^{-1}) Tween 20 (PBS-Tween 20) followed by incubation with a mouse monoclonal COX-2 or COX-1 antibody (dilutions 1 : 1000 and 1 : 2000, respectively) (kindly provided by Dr J. Maclouf, Paris) for 2 h at room temperature. The membranes were washed four times with PBS and finally incubated with a biotinylated second mouse IgG-antibody. COX-2 protein was detected by enhanced chemoluminescence (ECL-kit, Amersham, Braunschweig, Germany).

Determination of noradrenaline overflow into the coronary effluent

About 1 ml aliquots of the effluent were collected during the experiment according to the protocol (see below). The samples were stabilized with EDTA (5 mM) and stored at -20°C. Liquid-liquid-extraction of the samples (Smedes *et al*., 1982) was performed as previously described (Ehrenreich *et al*., 1997) using an extraction kit (Variokit, Biometra, Göttingen, Germany). In brief, to 0.5 ml of the sample, 20 μl (500 pg) dihydroxybenzoic acid (DHBA internal standard) and 150 μl of NH_4OH/NH_4Cl buffer, containing diphenylborate-ethanolamine and EDTA were added. To this mixture 750 μl of the extraction solvent, consisting of *n*-heptane containing *n*-octanol and tetraoctylammonium bromide were added, the sample was rocked for 2 min and centrifuged at $2000 \times g$ and 4°C temperature for 5 min. Next, 500 μl of the organic phase was transferred to another tube and mixed with 300 μl *n*-octanol and 60 μl 0.08 M acetic acid. The tube was again shaken for 2 min and centrifuged at $2000 \times g$ for 5 min. The lower (acetic acid) layer was then removed and stored in an Eppendorf tube at -4°C prior to separation by HPLC.

20 μl of the eluate was injected into the HPLC system. The column (125×3 mm) was packed with nucleosil with an average particle size of 3 μm. The mobile phase was a citrate buffer containing 6% methanol (Biometra, Göttingen, Germany). The pump (Merck 655 A-12) was adjusted to a flow rate of 0.4 ml min^{-1} at 140 bar. The electrochemical detector (Intro, Antec, Leiden, The Netherlands) was operated at 750 mV with an Ag/AgCl reference electrode and a thin-layer carbon-paste detector electrode. This HPLC system gave the following retention times: noradrenaline: 5.64 min, DHBA: 11.12 min.

Experimental protocol

After equilibration of the hearts, as seen from a stable contractile function and coronary vascular resistance, oxyhaemoglobin (3 μM) was added to the perfusion medium at time 0 and the perfusion was continued under otherwise identical conditions. At time 60 min, global normothermic ischaemia was induced by reduction of the coronary flow from 22 ml min^{-1} to 1.2 ml min^{-1}. Low-flow ischaemia was maintained for 120 min, i.e. until time 180 min. At this time, reperfusion was initiated by restoring the initial flow rate of 22 ml min^{-1} for another 60 min, i.e. until time 240 min. Control hearts were subjected to all of the same experimental procedures for the same period of time without ischaemia. We have previously shown that oxyhaemoglobin in the perfusion fluid did not affect ischaemia/reperfusion injury (Woditsch & Schrör, 1992).

The following groups of hearts were studied: ischaemic reperfused hearts and control hearts, perfused for the same period of time but without ischaemia. Both groups of hearts were either untreated or received 100 ng ml^{-1} nicotine (Sigma, Deisenhofen, Germany) which was added to the Krebs-Henseleit buffer. After it became apparent that nicotine caused a marked catecholamine overflow, an additional series of experiments was performed consisting of the same experimental groups and four additional groups treated with (−)pindolol. Pindolol was added to the perfusion medium 15 min prior to ischaemia at a concentration (3 μM) which was found to block completely the inotropic and chronotropic actions of 40 nM isoprenaline (not shown).

Statistics

The data are mean ± s.e.mean of n independent measurements. Statistical analysis was performed by one way ANOVA, followed by Bonferroni's multiple comparison tests. P levels of <0.05 were considered significant.

Results

Myocardial and coronary function

There were no differences with respect to LVEDP, LVSP, CPP and HR after stabilization of the preparations prior to ischaemia (not shown). Introduction of ischaemia was associated with a marked drop in LVSP and a slowly increasing LVEDP. Restoration of perfusion produced some recovery of the LVEDP while LVSP, CPP and HR remained unchanged and LVSP returned to control levels. Stable values in these parameters were obtained at 30 min of reperfusion and remained unchanged until the end of the experiment.

Administration of nicotine (100 ng ml^{-1}) did not cause any significant changes of myocardial function in non-ischaemic hearts. There was also no change by nicotine of HR and CPP in non-ischaemic hearts but a tendency for increase in reperfused ischaemic preparations ($P>0.05$). Nicotine significantly aggravated ischaemic myocardial dysfunction as seen from a doubling of the LVEDP. These data are summarized in Table 1.

Biochemical markers of myocardial tissue injury

The severity of myocardial tissue injury was assessed in terms of two biochemical markers – the efflux of creatine kinase and of troponin T into the coronary effluent, two established markers of the loss of myocellular integrity in myocardial ischaemia.

In non-ischaemic hearts, there was only a small efflux of CK-specific activity into the coronary effluent. In reperfused ischaemic hearts, this loss of CK was increased 5–6 fold, indicating a significant myocellular injury and the loss of large intracellular proteins. Nicotine caused a small, though

significant, CK loss in non-ischaemic hearts. However, in reperfused ischaemic preparations, the loss of CK was considerably enhanced and was more than doubled in ischaemic hearts in the presence of nicotine (Figure 1).

Similar results were obtained with troponin T (TnT) ($n=3-4$). There was no detectable release of this marker into the effluent from non-ischaemic hearts but a significant activity, amounting to 1.4 ± 0.2 U ml^{-1} during reperfusion after ischaemia. Nicotine alone did not affect TnT release in non-ischaemic preparations but increased TnT release almost 3 fold after ischaemia to 3.4 ± 0.1 U ml^{-1}, i.e. to three times the level seen in the absence of nicotine ($P < 0.05$).

Noradrenaline release

The data so far suggested that nicotine exerts only small effects by its own in non-ischaemic conditions but markedly increased ischaemic tissue injury. A possible explanation for this could be an enhanced catecholamine overflow. As already seen with myocardial function, there was no significant effect by nicotine on catecholamine release in non-ischaemic conditions while ischaemia was followed by the expected increase in noradrenaline overflow during early reperfusion. However, this overflow was stimulated about 5 fold in the presence of nicotine. The peak noradrenaline concentrations, measured after 2 min of reperfusion, were at least 10 fold higher as compared to non-nicotine treated ischaemic hearts. These data are summarized in Table 2 and Figure 2.

Prostacyclin release

Determination of 6-oxo-$PGF_{1\alpha}$ in the coronary effluent of reperfused ischaemic hearts confirmed the previous findings (Woditsch & Schrör, 1992) of a markedly increased prostacyclin release in early reperfusion and a small but significant stable increase in PGI_2 formation at the end of the reperfusion period. There was no change in 6-oxo-$PGF_{1\alpha}$ release in non-ischaemic hearts and no effect of nicotine in these preparations. However, nicotine significantly increased 6-oxo-$PGF_{1\alpha}$ release in ischaemic preparations (Figure 3).

Expression of COX-2 in ischaemic and non-ischaemic hearts

There was no expression of the COX-2 gene in non-ischaemic hearts in the absence but a small expression in the presence of nicotine. Ischaemia induced a significant gene activation of COX-2 as seen from the appearance of a 420 bp signal. This effect was markedly enhanced by nicotine. Original PCR experiments are shown in Figure 4. COX-2 and COX-1 protein levels are documented in the Western blots, shown in Figure 5. There was no significant change in COX-1 protein, no basal expression of COX-2 protein was found in untreated non-ischaemic hearts but a significant expression after ischaemia. The ratio COX-2 : COX-1 was used as an estimate of COX-2

Table 1 Left ventricular end-diastolic (LVEDP) and systolic (LVSP) pressures, coronary perfusion pressure (CPP) and heart rate (HR) in the different groups of hearts at 30 min of reperfusion

Group	n	*LVEDP* (mm Hg)	*LVSP* (mm Hg)	*CPP* (mm Hg)	*HR* (beats min^{-1})
		No ischaemia			
No nicotine	7	3.0 ± 0.8	74 ± 3	50 ± 5	149 ± 7
Nicotine	11	2.5 ± 1.1	74 ± 2	55 ± 6	167 ± 8
		Ischaemia			
No nicotine	14	16.0 ± 2.2*	73 ± 5	60 ± 5	125 ± 7
Nicotine	13	28.0 ± 4.0*†	77 ± 5	75 ± 4	145 ± 10

Data are mean$\pm$s.e.mean of n different heart preparations. The concentration of nicotine was 100 ng ml^{-1}. *$P<0.05$ (ischaemia *vs* no ischaemia). †$P<0.05$ (nicotine *vs* no nicotine).

Table 2 Noradrenaline concentration (ng l^{-1}) in coronary effluents from rabbit hearts during the first 2 min of reperfusion of ischaemic hearts or the corresponding time (180–182 min) in non-ischaemic hearts

Group	n	*No ischaemia*	n	*Ischaemia*
No nicotine	4	10 ± 1	5	251 ± 72*
Nicotine	2	14 ± 3	5	2989 ± 697*†

Data are mean$\pm$s.e.mean of n different heart preparations. The concentration of nicotine was 100 ng ml^{-1}. *$P<0.05$ (ischaemia *vs* no ischaemia). †$P<0.05$ (nicotine *vs* no nicotine).

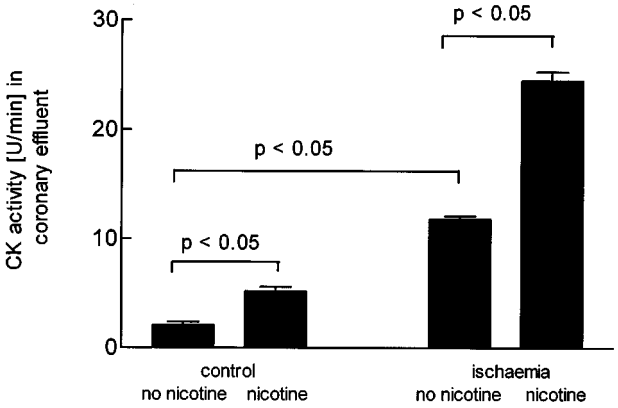

Figure 1 Creatine phosphokinase (CK)-specific activity in the coronary effluent at 30 min of reperfusion. The figure shows non-ischaemic and ischaemic hearts with nicotine or without nicotine treatment. The data are mean$\pm$s.e.mean of 7–12 different heart preparations per group.

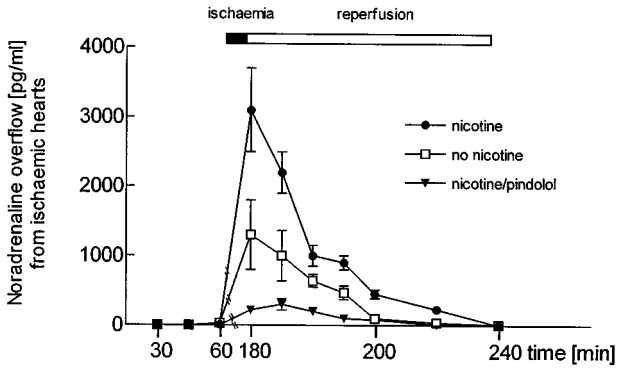

Figure 2 Time-course of noradrenaline overflow into the coronary effluent of ischaemic hearts. The hearts were either untreated (no nicotine) or received nicotine or nicotine plus pindolol. The data are mean$\pm$s.e.mean of 7–12 different heart preparations per group. Note that the time scale on the x axis is not linear.

protein expression and was found to be 0.42 ± 0.10 ($n=8$) in ischaemic hearts. Nicotine stimulated COX-2 expression to 0.21 ± 0.01 in non-ischaemic but 0.71 ± 0.10 in ischaemic hearts ($n=7$; $P<0.05$).

Effects of pindolol

Addition of the β-blocker pindolol prior to ischaemia caused a small but significant increase in heart rate in these spontaneously beating preparations from 125 ± 3 to 153 ± 7 b min^{-1} ($n=7-14$; $P<0.05$), probably due to its intrinsic sympathomimetic activity. Pindolol prevented the nicotine-induced increase in CPP in ischaemic hearts but did not modify any other parameter of cardiac function in ischaemic and non-ischaemic preparations. Specifically, there was no significant reduction of the elevated LVEDP in ischaemic hearts, amounting to 17.0 ± 2.2 mm Hg^{-1} in the absence and to 25.3 ± 2 mm Hg^{-1} in the presence of nicotine as compared to 16.0 ± 2.2 and 28.0 ± 4.0 mm Hg^{-1}, respectively, in non-pindolol-treated ischaemic hearts (c.f. Table 1). These values were not different from non-pindolol treated controls ($P>0.05$). In contrast, pindolol completely prevented the loss of CK-specific activity, amounting to only 4 ± 3 and 5 ± 3 U ml^{-1} in the effluent of reperfused ischaemic hearts in the absence and presence of nicotine, respectively. These values were not different from non-ischaemic controls ($P>0.05$; Figure 6).

Pindolol significantly suppressed the nicotine-induced increase in the COX-2 message to the level of either nicotine or ischaemia alone (not shown). This was paralleled by a complete prevention of the nicotine-induced COX-2 protein expression in ischaemic hearts. The COX-2 : COX-1 ratio amounted to 0.421 in ischaemic hearts in the absence, and to 0.711 in ischaemic hearts in the presence of nicotine, respectively. The corresponding numbers for pindolol-treated preparations were 0.128 in the absence and 0.131 in the presence of the β-blocker ($n=2$ per group). These results as well as the substantial reduction of the enhanced 6-oxo-$PGF_{1\alpha}$ and noradrenaline release in nicotine-treated hearts by pindolol are demonstrated in Figures 2 and 7, respectively.

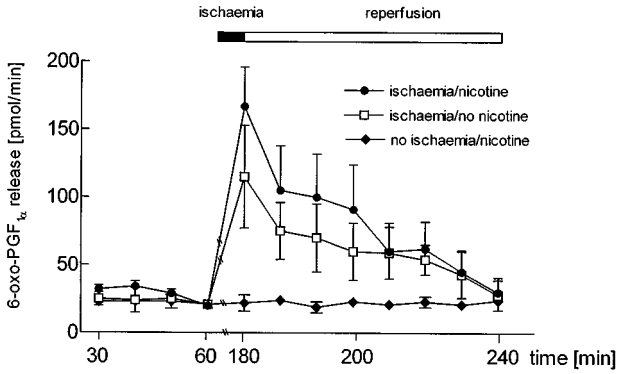

Figure 3 Time-course of 6-oxo-$PGF_{1\alpha}$ levels in the coronary effluent of ischaemic and non-ischaemic hearts with and without nicotine treatment. The data are mean $\pm$ s.e.mean of 7–12 different heart preparations per group. Note that the time scale on the x axis is not linear.

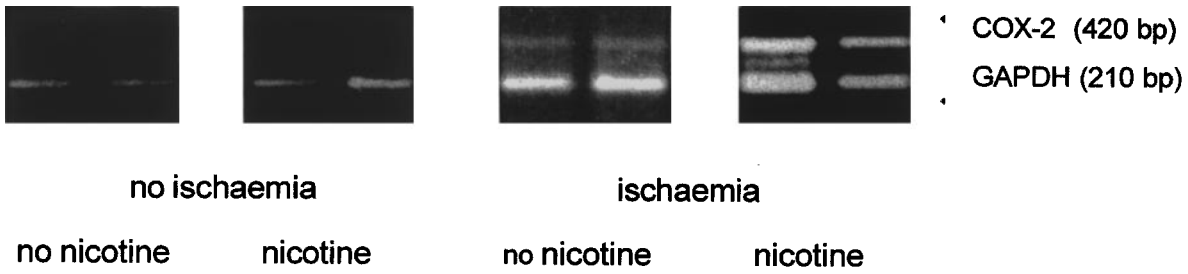

Figure 4 Polymerase chain reaction (PCR) of cyclooxygenase (COX-2) in rabbit hearts. Gyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal standard. Each group of treatment is represented by original mRNA preparations from two different hearts out of five per group with similar results.

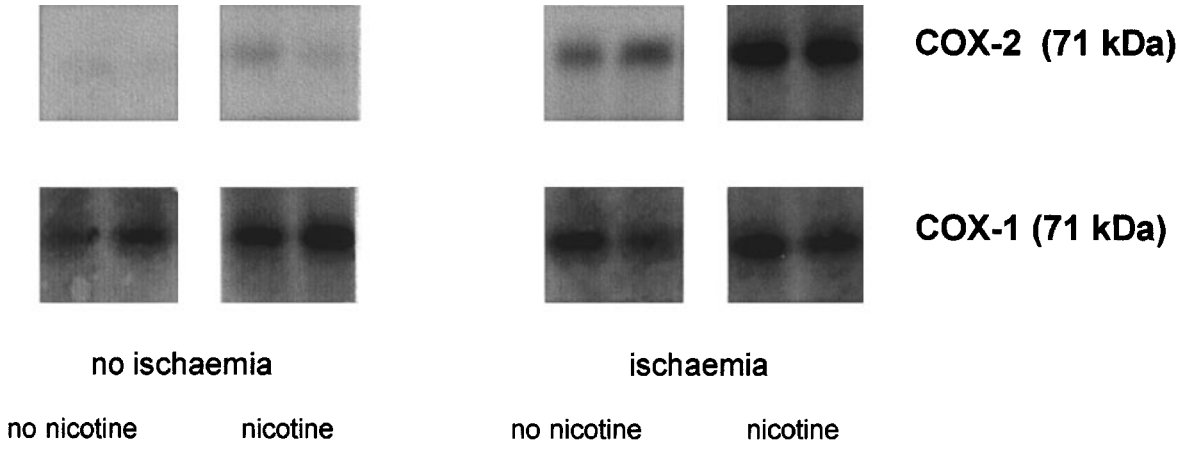

Figure 5 Western blot analysis of cyclooxygenase-1 and -2 in left ventricular tissue. The protein was detected by enhanced chemoluminescence. Each group of treatment is represented by original Western blots of two hearts out of five with similar results.

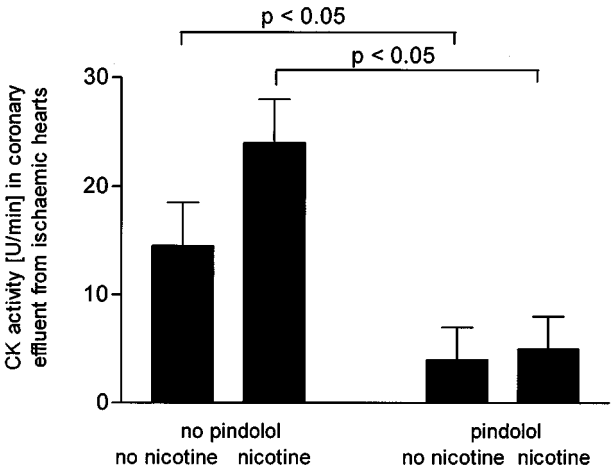

Figure 6 Creatine phosphokinase (CK)-specific activity in the coronary effluent of ischaemic hearts at 30 min of reperfusion. The figure demonstrates the effect of pindolol on ischaemic heart preparation with and without nicotine treatment. The data are mean ± s.e.mean of 7–12 different heart preparations per group.

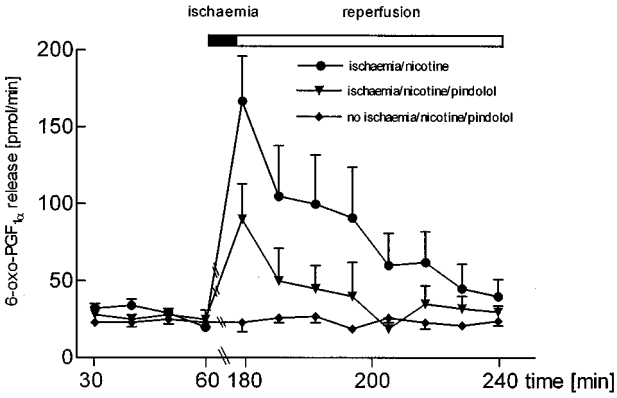

Figure 7 Time-course of 6-oxo-$PGF_{1\alpha}$ release in the coronary effluent of ischaemic and non-ischaemic hearts in the presence of nicotine and its modification by pindolol. The data are mean ± s.e.mean of 7–12 different heart preparations per group.

Discussion

Smoking of a single cigarette yields venous plasma nicotine concentrations of 5–30 ng ml^{-1} but 2–10 times higher peak concentrations are observed in arterial blood (Henningfield *et al.*, 1993), probably due to the immediate entry of nicotine into the arterial circulation, specifically the coronary circulation, after uptake by the lung. Effeney (1990) has shown that one week infusion of nicotine to rabbits yields blood levels of 50 ng ml^{-1} and twice as high levels in cardiac tissue. Concentrations of nicotine <1 μM (160 ng/ ml^{-1}) were previously found not to affect mechanical performance, prostaglandin release or noradrenaline overflow in isolated non-ischaemic rabbit hearts (Wennmalm, 1982). This was confirmed in the present trial for 'physiological' concentrations of nicotine in the perfusion medium.

This picture was changed completely in ischaemic heart preparations in which nicotine (100 ng ml^{-1}) considerably enhanced myocardial ischaemic injury as seen from the doubling of LVEDP and the considerable increase in loss of large intracellular proteins, such as CK and TnT, demonstrating significant myocellular injury. Clearly, nicotine has many actions which could account for these effects. This includes changes in vessel tone, increase in platelet reactivity and cardiac function. The present study was conducted under well-controlled *in vitro* conditions, excluding platelet-related phenomena as well as CNS-mediated effects of nicotine. There was a tendency for an increase in coronary vascular resistance by nicotine in reperfused ischaemic hearts but not in non-ischaemic preparations, confirming *in vivo* data on increase in coronary vessel tone by nicotine in patients suffering from coronary artery disease (Quillen *et al.*, 1993).

Several clinical studies have shown that smoking increases plasma catecholamine levels (Cryer *et al.*, 1976; Winniford *et al.*, 1986). It has also previously been demonstrated that myocardial ischaemia causes a marked increase in noradrenaline overflow (Wennmalm, 1977; Schrör *et al.*, 1981a), probably due to a redistribution of noradrenaline from its storage sites in adrenergic nerve terminals into the extraneuronal space (Holmgren *et al.*, 1981; Schrör *et al.*, 1981a; Muntz *et al.*, 1984) which is initiated by enhanced exocytosis at short-term ischaemia (Schömig *et al.*, 1984). This process is enhanced by nicotine (Schömig *et al.*, 1987; Krüger *et al.*, 1995). The present study confirms this and shows additionally that in the presence of nicotine, there is a marked, peak tenfold, increase in catecholamine release from ischaemic hearts. Treatment with the β-blocker pindolol prevented these deleterious effects. These data suggest that nicotine potentiates myocardial ischaemic injury and that the enhanced catecholamine levels might be a causative factor.

Another previous finding was that the severity of myocardial ischaemic injury is markedly enhanced by indomethacin, i.e. inhibition of endogenous prostaglandin synthesis (Schrör & Funke, 1985). We have also demonstrated protective actions of exogenously applied prostacyclins, namely iloprost, suggesting that the released PGI_2 had a tissue-protective effect (Schrör *et al.*, 1981a). In the present study, administration of nicotine not only resulted in a significant increase in noradrenaline overflow but also in enhanced prostaglandin formation. This was documented by a significant increase in PGI_2 production as assessed from 6-oxo-$PGF_{1\alpha}$ measurements in the coronary effluent. The total amount of PGI_2 released from ischaemic, nicotine-treated hearts was about twice as high as compared to effluents from non-nicotine treated controls. As a possible explanation, we demonstrate the expression of COX-2 protein after stimulation of the COX-2 gene while COX-1 protein remains unchanged. To the best of our knowledge, this is the first time that COX-2 expression has been described in the ischaemic heart and has been shown to be stimulated by nicotine.

There are several explanations why nicotine could induce COX-2 expression. The promoter region of the COX-2 but not COX-1 gene contains several enhancing elements, including a cyclic AMP response element (CRE). Recent studies have shown that this site is required for transcription of the COX-2 gene in vascular cells (Inoue *et al.*, 1995). Thus, the increase in tissue cyclic AMP levels, subsequent to extraneuronal catecholamine accumulation (Schrör *et al.*, 1981a; 1982), might cause upregulation of the COX-2 gene as well as aggravate myocardial tissue injury. Consequently, treatment with the β-blocker not only reduced the nicotine-induced COX-2 expression to the level of non-nicotine treated ischaemic hearts, suggesting that this response was β-receptor mediated and related to the severity of ischaemic injury.

Wu *et al.* (1995) have recently shown that lysophospholipids are potent stimuli for COX-2 expression in vascular cells. Lysophospholipids are generated during phospholipase-induced destruction of cell membranes. Kolbeck-Rühmkorff *et*

al. (1993) have shown that infusion of isoprenaline to ischaemic hearts results in c-*fos* expression. We have also found enhanced c-*fos* mRNA levels in nicotine-treated ischaemic hearts (not shown), suggesting that the AP2 region in the COX-2 promoter region (Inoue *et al*., 1995) might also be involved.

This raises the question, whether COX-2 expression in reperfused ischaemic hearts by nicotine is a consequence of enhanced tissue injury or in some way directly mediated by nicotine. This is currently difficult to answer. Since nicotine effects on catecholamine release are short-acting and subject to tachyphylaxis within a few minutes (Wennmalm 1978a,b; Löffelholz, 1979) it is difficult to explain a long-term stimulation. It should be noted, however, that most of the previous trials have used considerably larger concentrations of nicotine, up to 100 μM which are far above nicotine levels in smokers which are in the nanomolar range. As discussed before, the prevention of COX-2 expression in relation to prevention of ischaemic injury by pindolol would favour the concept of enhanced tissue injury as a cause of enhanced COX-2 expression.

Nowak and colleagues (1987) have published that cigarette smoking, yielding plasma levels of nicotine amounting to about 30 ng ml^{-1}, resulted in significantly elevated thromboxane and prostacyclin levels in circulating blood as determined from the renal excretion of index metabolites. Low-dose aspirin (20 mg bid) normalized thromboxane excretion to that of non-smoking controls. However, there was only a minor reduction of the smoking-related elevated prostacyclin excretion. Aspirin is known to be a rather selective COX-1 inhibitor (Mitchell *et al*., 1993), an effect particularly prominent at low doses. Our data would suggest that this failure of aspirin to block nicotine-induced prostacyclin formation is due to its small inhibitory capacity against COX-2.

In conclusion, the present data demonstrate that nicotine aggravates myocardial ischaemic injury. This is accompanied by transcriptional upregulation of COX-2, eventually resulting in enhanced prostacyclin generation. While this effect appears to be of minor importance for the ischaemic tissue, possibly because it is too small to exert cardioprotective effects, it could nevertheless result in inhibition of platelet function, thereby contributing to improved lysis of platelet-thrombi *in vivo*.

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