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Formation of 8-iso-$PGF_{2\alpha}$ and thromboxane A_2 by stimulation with several activators of phospholipase A_2 in the isolated human umbilical vein

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1 We investigated the effects of the phospholipase A_2 (PLA_2) activators calcium ionophore A 23187, hydrogen peroxide (H_2O_2), bradykinin (BK), histamine and noradrenaline (NA) on the 8-iso-prostaglandin (PG)$F_{2\alpha}$ formation in the isolated human umbilical vein and the isolated rabbit ear. For comparison, the influence of these substances on the thromboxane A_2 (TXA_2) release was also investigated. The release of total (esterified as well as free) 8-iso-$PGF_{2\alpha}$, free 8-iso-$PGF_{2\alpha}$ and TXB_2, the stable metabolite of TXA_2, was determined by specific enzyme immunoassays.

2 The results show that bolus injections of 5.4 mmol H_2O_2, 30 nmol A 23187, 10 nmol BK, 50 nmol histamine and 20 nmol NA caused an increased release of total 8-iso-$PGF_{2\alpha}$ in the umbilical vein and the rabbit ear. A perfusion with H_2O_2 at a final concentration of 0.3 mM also increased the release of this isoprostane. Increased formation of free 8-iso-$PGF_{2\alpha}$ was induced by A 23187 injection and by both modes of H_2O_2 administration, but not by the other treatments.

3 Bolus injections of A 23187, BK and histamine induced an increased release of TXB_2 in both organs. Both modes of H_2O_2 administration and NA showed no releasing effects.

4 In conclusion, our results show that the substances used are able to stimulate the formation of 8-iso-$PGF_{2\alpha}$ concurrently with the release of PGs. This effect might be of pathophysiological relevance in inflammatory and cardiovascular diseases in which an enhanced release of free radicals, BK, histamine or NA play an important role.

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Abbreviations: A 23187, calcium ionophore A 23187; BK, bradykinin; $cPLA_2$, cytosolic phospholipase A_2; H_2O_2, hydrogen peroxide; NA, noradrenaline; PG, prostaglandin; PL, phospholipase; $sPLA_2$, secretory phospholipase A_2; TX, thromboxane

Introduction

The isoprostane 8-iso-$PGF_{2\alpha}$ is formed by free radical-catalyzed peroxidation of arachidonic acid independent of cyclo-oxygenase *in vivo* and *in vitro* (Morrow *et al*., 1990a,b; 1994). Reactive free radicals play an important role in the pathophysiology of a wide spectrum of disorders including atherosclerosis, ischaemia-reperfusion injury, inflammatory diseases, cancer and aging (Wallace, 1997). Increased formation of 8-iso-$PGF_{2\alpha}$ was detected in human atherosclerotic lesions and plaques (Pratico *et al*., 1997; Delanty *et al*., 1997), in patients with heart failure (Mallat *et al*., 1998) or in patients with hypercholesterolemia (Devi *et al*., 1997; Palombo *et al*., 1999). Eicosanoids also play an important role in the pathogenesis of various diseases like inflammatory or cardiovascular diseases (Davie & Macintyre, 1992; Sinzinger *et al*., 1990).

Mediators such as bradykinin (BK) and histamine or oxygen free radicals and catecholamines are involved in inflammation and cardiovascular diseases (Vane & Ferreira, 1978; Jean & Bodinier, 1994; Kopin, 1989). It is well known that BK, histamine, noradrenaline (NA), the divalent cation ionophore A 23187 and hydrogen peroxide (H_2O_2) activate phospholipase A_2 (PLA_2) by calcium-mobilization followed by an increased release of eicosanoids (Pace-Asciak & Rangraj, 1977; Cherouny *et al*., 1988; Rao *et al*., 1995; Schoenberg, 1997; Kajiyama *et al*., 1990; Reddy *et al*., 1995; Kennedy *et al*., 1996; Förstermann *et al*., 1984; Weigel *et al*., 1991; Juan, 1979; Juan & Sametz, 1980; Sametz & Juan, 1982).

The aim of this study was to investigate whether, in addition to PG release, these substances also stimulate formation of 8-iso-$PGF_{2\alpha}$ in the isolated perfused human umbilical vein and for comparison in the isolated perfused rabbit ear, a model for a peripheral vascular system. Since isoprostanes caused their vasoconstrictor effects by activation of the thromboxane receptor (Takahashi *et al*., 1992), we determined the release of thromboxane B_2 (TXB_2) in both organs.

Method

Organ preparation

Isolated perfused human umbilical vein Umbilical cords collected immediately after delivery were transported to the laboratory in an ice cold Tyrode solution previously aerated with a mixture of 95% O_2 and 5% CO_2. A segment with a length of 10 cm was cut from the median division of each cord. After flushing intraluminally the vein was cannulated and connected to a peristaltic pump (Gilson, Minipuls 3). Perfusion

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with Tyrode solution (37°C, gassed with 95% O_2 and 5% CO_2) was adjusted to 3 ml min^{-1}. All experiments were started 1–3 h after delivery.

Isolated perfused rabbit ear Rabbits of either sex (2.5–3.5 kg body weight; Department of Biomedical Research, Section Animal Facility, Graz) were sacrificed by an overdose of pentobarbitone (>50 mg kg^{-1} i.v.). The ears were cut off, their central artery cannulated and connected to a peristaltic pump (Gilson, Minipuls 3). Perfusion with Tyrode solution (37°C, gassed with 95% O_2 and 5% CO_2) was adjusted to 3 ml min^{-1}.

Experimental design

After an equilibration period of 60 min, the calcium-ionophore A 23187 (30 nmol), BK (10 nmol), histamine (50 nmol) and NA (20 nmol) were injected as a bolus into the central artery of the rabbit ear or into the human umbilical vein. These doses were chosen for stimulation of PG release, as described (Juan, 1979; Juan & Sametz, 1980; Griesbacher *et al*., 1997; Förstermann *et al*., 1984). H_2O_2 was given primary as a perfusion at a final concentration of 0.3 mM, which is able to stimulate PG release (Cherouny *et al*., 1988). For comparison with the mode of application of the other substances used, the complete amount of H_2O_2 perfused within 6 min (5.4 mmol) was injected as a bolus in four further experiments. The injection volume of A 23187 dissolved in ethanol was 30 μl and that of the other substances dissolved in NaCl (0.9%) 100 μl.

The outflow was collected in 2 min samples (6 ml) one before and three after bolus injections of the substances or after the start of perfusion with H_2O_2. For determination of total (esterified as well as free) 8-iso-$PGF_{2\alpha}$ release, hydrolysis of phospholipid esterified 8-iso-$PGF_{2\alpha}$ was performed by adding one part of 8 N NaOH to three parts of the liquid sample and heating at 45°C for 2 h. After cooling, the mixture was neutralized with an equal volume of 2N HCl. For determination of free 8-iso-$PGF_{2\alpha}$ and TXA_2 release, 1 ml of each sample was extracted three times with an equal volume of ethyl acetate, evaporated and redissolved in assay buffer.

8-iso-$PGF_{2\alpha}$ and TXA_2, measured as its stable metabolite TXB_2, were determined by using sensitive and specific total (esterified as well as free) 8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ (free) and TXB_2 Enzyme-Immunoassay (EIA) kits (Assay Designs, Inc., Ann Arbor, MI, U.S.A.). Four experiments were performed for each substance.

Materials

Bradykinin acetate, calcium-ionophore A 23187, histamine dihydrochloride, noradrenaline bitartrate were purchased from Sigma (Vienna, Austria) and hydrogen peroxide (30%) from Merck (Vienna, Austria). Bradykinin, histamine and noradrenaline were dissolved and diluted in 0.9% saline freshly before experiments. The water insoluble ionophore A 23187 was dissolved in ethanol. All doses given refer to the free bases. The composition of Tyrode solution was (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.15, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.6.

Statistical analysis

The data are expressed as the mean±s.e.mean of four experiments for each substance. Statistical analysis was performed by Student's *t*-test for unpaired data. Probability values of $P<0.05$ were considered significant and illustrated in the appropriate figures by an asterisk.

Results

Release of 8-iso-$PGF_{2\alpha}$

Bolus injections of 30 nmol A 23187 (Figure 1A,B), 50 nmol histamine (Figure 2A,B), 5.4 mmol H_2O_2 (Figure 3A,B), 10 nmol BK (Figure 4A) and 20 nmol NA (Figure 4B) caused an increased and significant release of total 8-iso-$PGF_{2\alpha}$ in the umbilical vein as well as in the rabbit ear. The greatest release occurred within the first 2 min after H_2O_2 injections (Figure 3A,B), whereas after injections of all other substances used the greatest release occurred after the first 2 min (Figures 1A,B, 2A,B and 4A,B). Therefore, the latency of H_2O_2 for the 8-iso-$PGF_{2\alpha}$ release appears to be shorter than that of the other substances used. Thirty μl ethanol, the vehicle of A 23187, was without effect (data not shown). The total amount of 8-iso-$PGF_{2\alpha}$ in excess of the basal release was 4016 pg after A 23187,

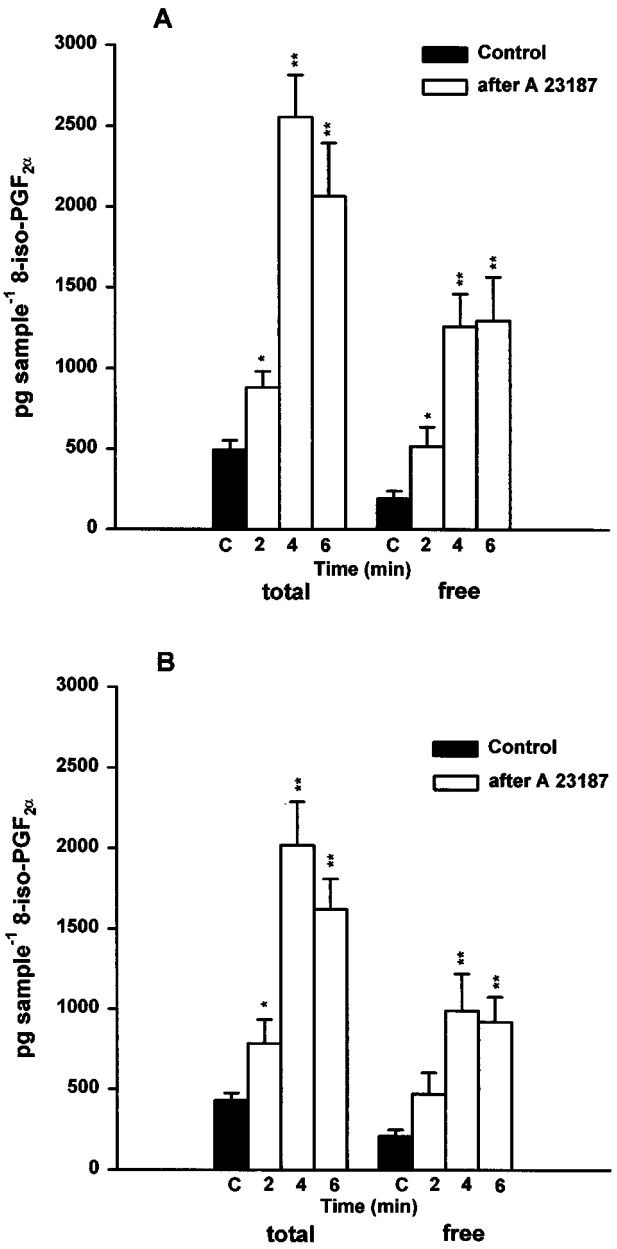

Figure 1 Release of total (left graph) and free (right graph) 8-iso-$PGF_{2\alpha}$ in pg $sample^{-1}$ (6 ml) induced by bolus injections of 30 nmol A 23187 in the isolated human umbilical vein (A) and the isolated rabbit ear (B). Vertical bars represent s.e.mean. Significance of difference from controls: $*P<0.05$, $**P<0.01$. $n=4$.

6237 pg after H_2O_2, 1488 pg after histamine, 1339 pg after BK and 1029 pg after NA injections in the umbilical vein and in the rabbit ear 3132, 4280, 2046, 1156 and 846 pg, respectively. A perfusion with H_2O_2 at a final concentration of 0.3 mM caused also a significantly increased release of total 8-iso-$PGF_{2\alpha}$ in both organs (Figure 5A,B). In this case the total amount of 8-iso-$PGF_{2\alpha}$ in excess of the basal release was 3853 pg in the umbilical vein and 2374 pg in the rabbit ear.

Significantly increased amounts of free 8-iso-$PGF_{2\alpha}$ could be determined after injections of A 23187 (Figure 1A,B, right graphs), H_2O_2 (Figure 3A,B, right graphs) and during H_2O_2 perfusions (Figure 5A,B, right graphs) in both organs. No increased amount could be measured after histamine injections (Figure 2A, B, right graphs), BK and NA (data not shown). The total amount of free 8-iso-$PGF_{2\alpha}$ in excess of the basal release was 2499 pg after A 23187, 1297 pg after H_2O_2 injections and 797 pg during H_2O_2 perfusions in the umbilical vein. The values obtained in the rabbit ear were 1750, 612 and 932 pg, respectively. From that, the following ratios of free to esterified 8-iso-$PGF_{2\alpha}$ could be calculated: after A 23197 injections 1 : 0.61, after H_2O_2 injections 1 : 3.8 and during H_2O_2 perfusions 1 : 3.6 in the umbilical vein and in the rabbit ear after A 23197 injections 1 : 0.78, after H_2O_2 injections 1 : 3.6 and during H_2O_2 perfusions 1 : 2.9.

The basal release of free 8-iso-$PGF_{2\alpha}$ was 192 ± 6 pg and that of total 8-iso-$PGF_{2\alpha}$ 407 ± 29 pg (ratio free to esterified 1 : 1.1) in the umbilical vein. In the rabbit ear the values for free 8-iso-$PGF_{2\alpha}$ was 242 ± 12 pg and that for total 8-iso-$PGF_{2\alpha}$ 480 ± 30 pg (ratio free to esterified 1 : 1). Bolus injections of 100 μl NaCl (0.9%), the vehicle of Bk, NA and histamine, and of 30 μl ethanol, the vehicle of A 23187, were without effects (data not shown).

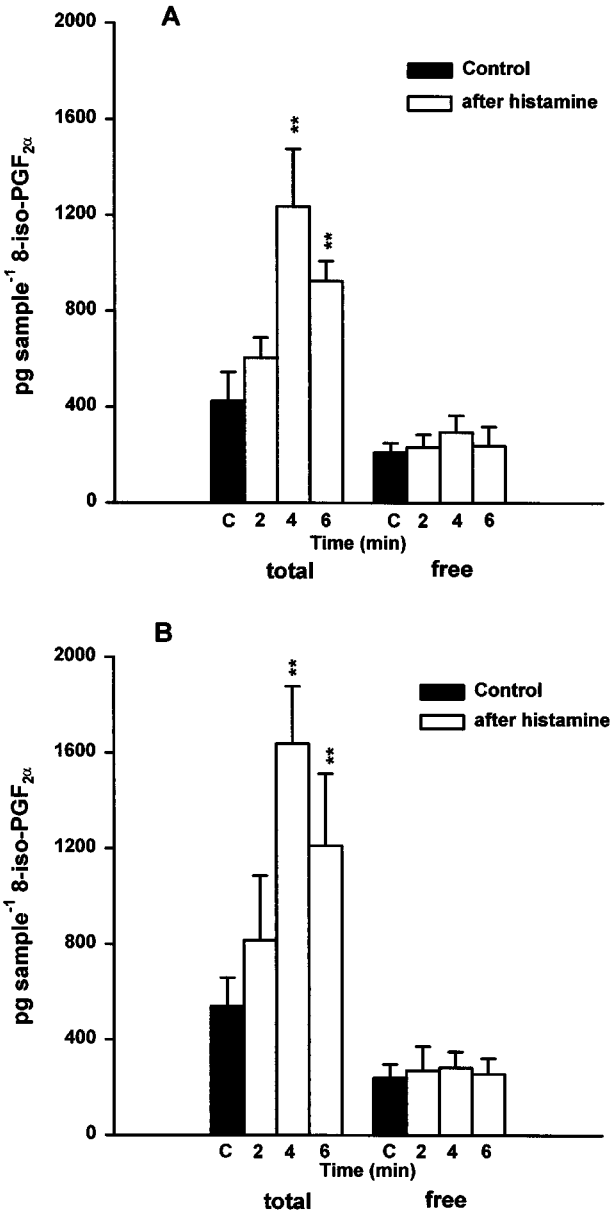

Figure 2 Release of total (left graph) and free (right graph) 8-iso-$PGF_{2\alpha}$ in pg $sample^{-1}$ (6 ml) induced by bolus injections of 50 nmol histamine in the isolated human umbilical vein (A) and the isolated rabbit ear (B). Vertical bars represent s.e.mean. Significance of difference from controls: $^{**}P<0.01$. $n=4$.

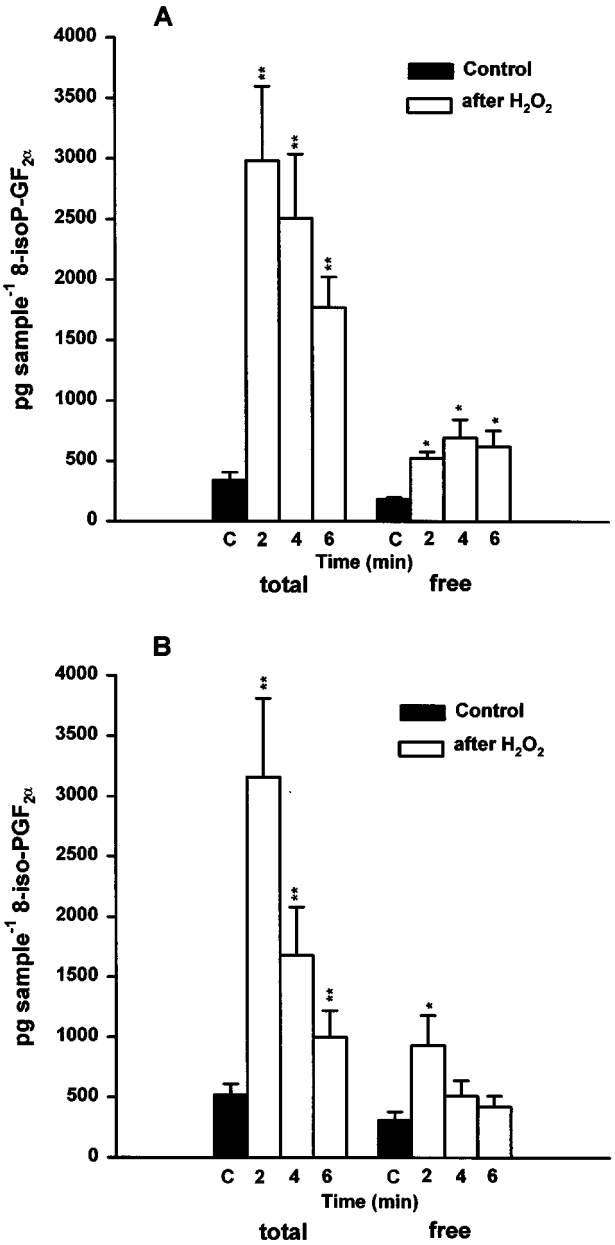

Figure 3 Release of total (left graph) and free (right graph) 8-iso-$PGF_{2\alpha}$ in pg $sample^{-1}$ (6 ml) induced by bolus injections of 5.4 mmol H_2O_2 in the isolated human umbilical vein (A) and the isolated rabbit ear (B). Vertical bars represent s.e.mean. Significance of difference from controls: $^{*}P<0.05$, $^{**}P<0.01$. $n=4$.

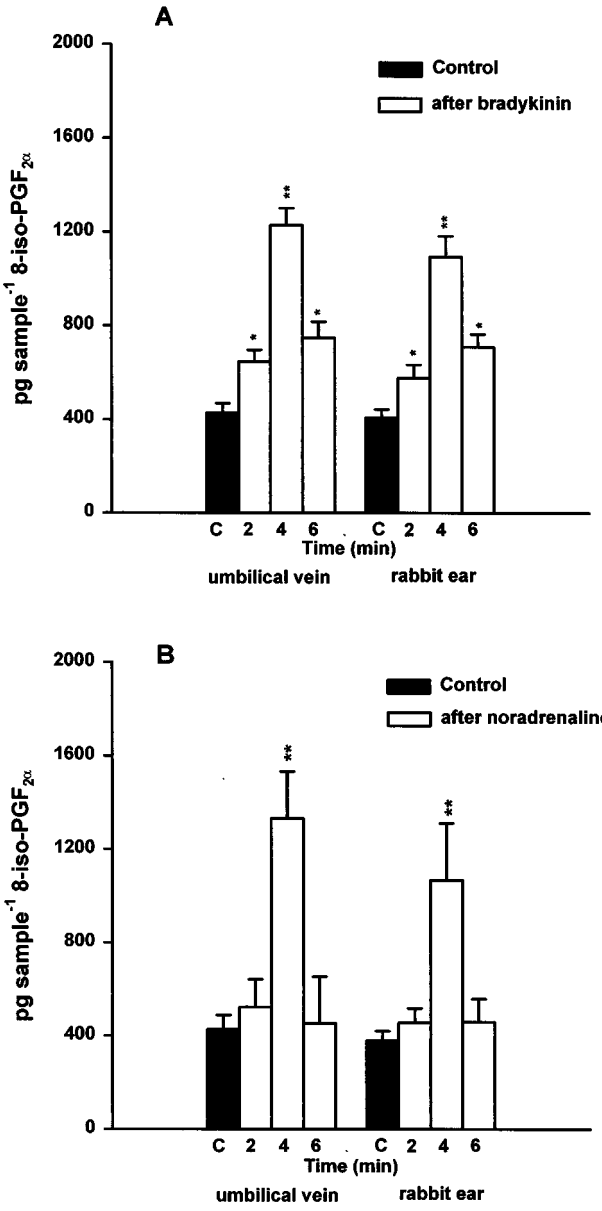

Figure 4 Release of total 8-iso-PGF$_{2\alpha}$ in pg sample^{-1} (6 ml) induced by bolus injections of 10 nmol bradykinin (A) and 20 nmol noradrenaline (B) in the isolated human umbilical vein (left graph) and the isolated rabbit ear (right graph). Vertical bars represent s.e.mean. Significance of difference from controls: $^*P<0.05$, $^{**}P<0.01$. $n=4$.

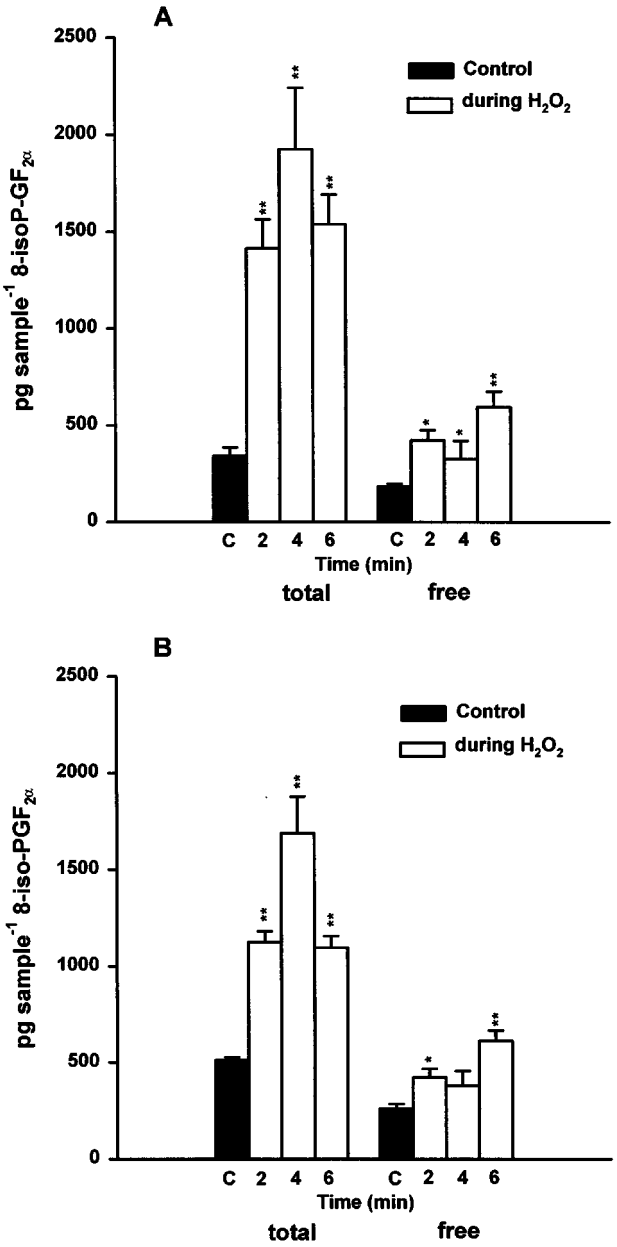

Figure 5 Release of total (left graph) and free (right graph) 8-iso-PGF$_{2\alpha}$ in pg sample^{-1} (6 ml) induced by perfusions with 0.3 mM H_2O_2 in the isolated human umbilical vein (A) and the isolated rabbit ear (B). Vertical bars represent s.e.mean. Significance of difference from controls: $^*P<0.05$, $^{**}P<0.01$. $n=4$.

Release of TXB$_2$

Bolus injections of 30 nmol A 23187 (Figure 6A), 50 nmol histamine (Figure 7A) and 10 nmol BK (Figure 7B) increased the release of TXB$_2$ in the umbilical vein as well as in the rabbit ear significantly. Injections of 5.4 mmol H_2O_2 (Figure 6B) and a perfusion at a concentration of 0.3 mM (data not shown) and also 20 nmol NA (data not shown) showed no effect in both organs. The total amount of TXB$_2$ in excess of the basal release was 2810 pg after A 23187, 347 pg after histamine and 258 pg after BK injections in the umbilical vein and in the rabbit ear 2103, 300 and 300 pg, respectively. Bolus injections of 100 μl NaCl (0.9%), the vehicle of BK, NA and histamine, and of 30 μl ethanol, the vehicle of A 23187, were without effects (data not shown).

Discussion

The results of the present study show that A 23187, H_2O_2, histamine, BK and NA caused an increased release of total 8-iso-PGF$_{2\alpha}$ in the isolated human umbilical vein and the isolated rabbit ear, whereby no significant difference between the two organs in relation to the release appeared. Increased formation of 8-iso-PGF$_{2\alpha}$ in diseases like atherosclerosis or inflammation provides an accurate and reliable indication of free radical catalyzed lipid peroxidation (Pratico, 1999). Free radicals (especially superoxide anion) and other oxygen species (such as H_2O_2) are continuously produced *in vivo* and the production increases e.g. in inflammatory and cardiovascular diseases (Halliwell *et al*., 1992). *In vitro* studies describe a release of superoxide anion from endothelial cells in response

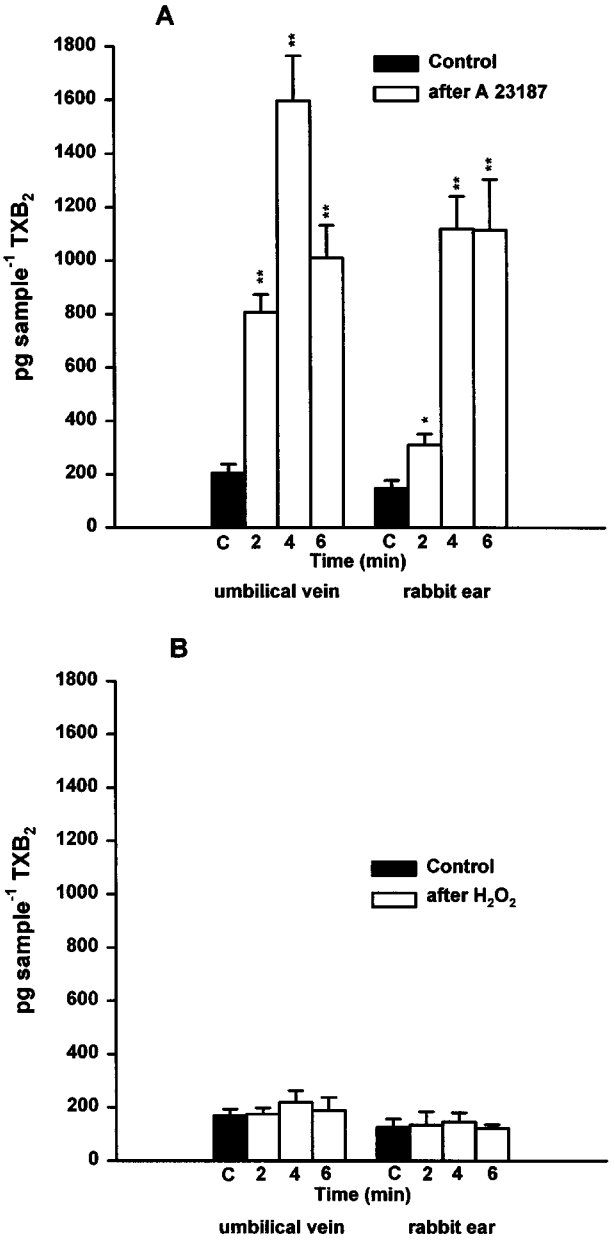

Figure 6 Release of total TXB_2 in pg sample^{-1} (6 ml) induced by bolus injections of 30 nmol A 23187 (A) and 5.4 mmol H_2O_2 (B) in the isolated human umbilical vein (left graph) and the isolated rabbit ear (right graph). Vertical bars represent s.e.mean. Significance of difference from controls: $^*P<0.05$, $^{**}P<0.01$.

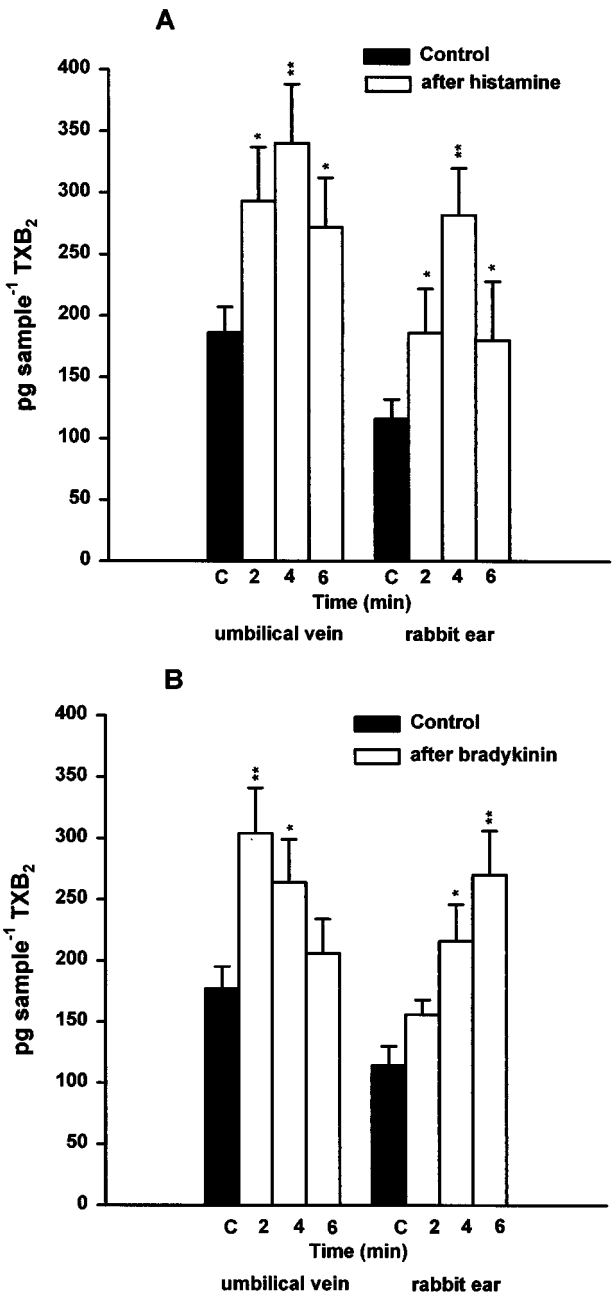

Figure 7 Release of total TXB_2 in pg sample^{-1} (6 ml) induced by bolus injections of 50 nmol histamine (A) and 10 nmol bradykinin (B) in the isolated human umbilical vein (left graph) and the isolated rabbit ear (right graph). Vertical bars represent s.e.mean. Significance of difference from controls: $^*P<0.05$, $^{**}P<0.01$.

to various substances such as BK or A 23187 (Matsubara & Ziff, 1986; Holland *et al*., 1990), which was decreased by cyclooxygenase inhibitors (Holland *et al*., 1990). Elevated intracellular calcium activates PLA_2 and PLC followed by stimulation of arachidonic acid metabolism, whose intermediates also generate free radicals (Kuehl *et al*., 1980; Kontos *et al*., 1985). All substances used activate PLs by calcium mobilization and subsequent eicosanoid release (Benbarek *et al*., 1999; Kajiyama *et al*., 1990; Reddy *et al*., 1995; Cherouny *et al*., 1988; Vercellotti *et al*., 1991; Rao *et al*., 1995; Schoenberg, 1997). Liu & Li (1995) proposed that formation of reactive oxygen species and arachidonic acid metabolites initiate feedback loops in which formation of one leads to generation of the others. Thus, elevated free radicals after administration of the agents used might be responsible for the increased formation of 8-iso-$PGF_{2\alpha}$. The short latency and the release of the greatest amount of 8-iso-$PGF_{2\alpha}$ by H_2O_2 injection might indicate an additional direct effect of this oxygen species.

Although the high doses of 5.4 mmol H_2O_2 did not increase the TXB_2 release in contrast to the other substances at nmol doses used, the possibility must be taken into account that these high doses also take part in the greater efficacy of H_2O_2 than the other drugs used. The more pronounced release induced by A 23187 than that of BK, histamine or NA might be lie in the different mechanism of calcium mobilization. The divalent cation ionophore A 23187 is known to activate PLs, the initial step of PG release, mainly by producing an influx of extracellular calcium into the cell and by the release of calcium from intracellular stores (Easwell & Pressman, 1972; Hainaut & Desmedt, 1974). However, histamine stimulates prostaglan-

din release by activation of H_1-receptor (Juan & Sametz, 1980) and BK of the B_2-receptor (Griesbacher *et al*., 1997) in the isolated rabbit ear. NA is able to stimulate the release of eicosanoids by activation of the α-adrenoceptors e.g. in rabbit platelets (Kajiyama *et al*., 1990). We can speculate that the lesser formation of 8-iso-$PGF_{2\alpha}$ stimulated by BK, histamine or NA than by A 23187 might be a consequence of a weaker calcium mobilization by receptor activation. But to clarify this, the influence of specific receptor antagonists and radical scavengers on the 8-iso-$PGF_{2\alpha}$ release should be investigated in a further study. Watkins *et al*. (1999) described a cyclo-oxygenase dependent release of 8-iso-$PGF_{2\alpha}$ induced by H_2O_2 in cultured human umbilical endothelial cells. Therefore, it is also necessary to investigate the influence of cyclo-oxygenase inhibitors on the 8-iso-$PGF_{2\alpha}$ release induced by the substances used in the present study.

It is assumed that 8-iso-$PGF_{2\alpha}$ esterified to phospholipids will be liberated to its free form enzymatically by PL(s) activity, probably by PLA_2 (Morrow *et al*., 1992). More than 50% of the total 8-iso-$PGF_{2\alpha}$ release induced by A 23187 could be determined as its free form as our results show, whereas after BK, histamine or NA injections no increased free 8-iso-$PGF_{2\alpha}$ release could be measured. The different mechanism of calcium mobilization probably responsible for different intensity of PL activity as discussed above might also influence the liberation of 8-iso-$PGF_{2\alpha}$ esterified to phospholipids.

TXA_2 can be released not only from platelets but also from endothelial cells, e.g. from human umbilical endothelial cells (Weigel *et al*., 1991). In the present study a stimulated release of TXA_2, measured as TXB_2, induced by A 23187, BK and histamine was obtained. However, H_2O_2 and NA at the doses used showed no effects. These different releasing effects might be also due to the different intensity of PLA_2 activation. The TXB_2 amounts in excess of the basal values were much lower than the amounts of 8-iso-$PGF_{2\alpha}$. These results suggest that endothelial cells are more sensitive for isoprostanes than for TXA_2 formation. Furthermore, it can be assumed that isoprostanes might be released concurrently with PGs after stimulation with the substances used, which can also be produced endogenously.

H_2O_2 at the doses used did not stimulate the release of TXB_2 but it liberated 8-iso-$PGF_{2\alpha}$ in its free form (about 20% of total release). On the other hand BK and histamine stimulated the TXB_2 release in similar amounts but not the liberation of free 8-iso-$PGF_{2\alpha}$. Two types of PLA_2, the secretory ($sPLA_2$) calcium independent and the cytosolic ($cPLA_2$) calcium dependent, have been described in inflammatory processes (Uhl *et al*., 1997; Cirino, 1998). It was found that $cPLA_2$ is of greater importance than $sPLA_2$ for release of arachidonic acid and its metabolites (Tibes *et al*., 1997; Bingham & Austen, 1999). In view of these findings, it is possible that H_2O_2 activates $sPLA_2$ rather than $cPLA_2$ and thus it liberated 8-iso-$PGF_{2\alpha}$ from phospholipids but did not stimulate the release of TXB_2 in both isolated organs used. To clarify this phenomenon, investigations with PL inhibitors are necessary. In conclusion, it can be presumed that in inflammatory and cardiovascular diseases, in which enhanced levels of BK, histamine and NA play an important role, 8-iso-$PGF_{2\alpha}$ will be released by these endogenous substances.

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