Endothelial-derived superoxide anions in pig coronary arteries: evidence from lucigenin chemiluminescence and histochemical techniques

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1. The generation of superoxide anions (O_2^-) by intact pig coronary artery rings was measured using a lucigenin-enhanced chemiluminescence technique and a histochemical technique with Nitroblue Tetrazolium (NBT) staining.

2. Isolated arteries with intact endothelium generated O_2^- at a rate of $9{\cdot}0 \pm 0{\cdot}8$ pmol min^{-1} (mg dry weight)$^{-1}$; this rate was diminished by about 24% when the endothelium was removed. The NBT staining of arterial ring preparations showed formazan precipitation mainly in the intima. Arterial rings were pretreated with diethylthiocarbamate in order to inhibit Cu–Zn superoxide dismutase (SOD) activity which increased the O_2^- generation by $184 \pm 55\%$ ($n = 10$; $P < 0{\cdot}01$). Stimulation of protein kinase C with phorbol 12-myristate 13-acetate (5 μM) enhanced endothelium-dependent O_2^- generation by $136 \pm 20\%$ ($n = 19$; $P < 0{\cdot}01$). Neither stimulation with bradykinin or substance P, nor inhibition with N^G-nitro-L-arginine methyl ester of endothelial nitric oxide synthase had a significant effect on O_2^- generation. In contrast, the inhibition of flavoproteins with diphenyliodonium decreased concentration-dependent O_2^- generation (IC_{50}, $1{\cdot}85 \pm 5{\cdot}33$ μM). Inhibition of tetrahydrobiopterin synthesis with 2,4-diamino-6-hydroxy-pyrimidine resulted in a reduced generation of O_2^- by about 55%.

3. The addition of 100 μM NADH and 100 μM NADPH resulted in an excessive generation of O_2^- at a rate of $0{\cdot}68 \pm 0{\cdot}03$ and $0{\cdot}26 \pm 0{\cdot}01$ nmol O_2^- min^{-1} (mg protein)$^{-1}$, respectively, in the membrane fraction, but not in the cytosolic fraction, of homogenates obtained from arteries.

4. The results suggest that intact coronary arteries do generate O_2^- under basal conditions and that the endothelial layer significantly contributes to this phenomenon. This generation of O_2^- is greatly influenced by intrinsic SOD activity. It is suggested that basal vascular O_2^- generation is mainly due to membrane-bound NAD(P)H oxidase activity and/or tetrahydrobiopterin-dependent processes.

The synthesis of nitric oxide (NO) by vascular endothelial cells is responsible for the local regulation of vascular tone (Moncada & Higgs, 1993). In addition, it has been hypothesized that the NO pathway is involved in various protective functions of intact endothelium, including the inhibition of platelet and leucocyte adhesion, and the inhibition of smooth muscle cell proliferation (Moncada & Higgs, 1993). NO is synthesized in endothelial cells from the amino acid L-arginine by a calcium–calmodulin-regulated enzyme utilizing the cofactors NADPH, flavins and tetrahydrobiopterin (the cofactor for NO synthase (NOS)) (Moncada & Higgs, 1993).

In addition to NO, endothelial cells may produce superoxide anions (O_2^-) under certain conditions. Rosen & Freeman (1984) were the first to describe the fact that cultured porcine aortic endothelial cells produce intra- and extracellular O_2, as detected by electron paramagnetic resonance spectroscopy. Using cytochrome *c* as a detector for O_2^-, an increased production of radicals has been demonstrated for human umbilical vein endothelial cells (HUVEC) in response to the calcium ionophore A23187, phorbol esters and cytokines (Matsubara & Ziff, 1986*a*,*b*). Holland, Pritchard, Pappolla, Wolin, Rogers & Stemerman (1990) demonstrated an increased release of O_2^- from HUVEC in response to

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bradykinin that could be partially prevented by the cyclo-oxygenase inhibitor indomethacin. Heim, Thomas & Ramwell (1991) measured a basal release of O_2^- from intact rabbit aortic rings that could be enhanced after pre-incubation with the compound alloxan; mechanical removal of the endothelium did not affect the basal release of O_2^-, but reduced the alloxan-stimulated release by about 50%. Laurindo *et al.* (1994) found a flow-dependent release of radical adducts in the perfused rabbit aorta that could be blocked by either the mechanical removal of the endothelium or by the addition of the O_2^- scavenger Cu–Zn superoxide dismutase (SOD), suggesting an endothelial, flow-dependent release of O_2^-. Finally, endothelial generation of O_2^- is believed to be responsible for the free radical peroxidation of low-density lipoproteins, a major event in the pathogenesis of atherosclerosis (Steinbrecher, 1987).

In contrast to these studies, other investigators have failed to demonstrate the endothelial release of O_2^- in intact vessels. Pagano, Tornheim & Cohen (1993) measured the release of O_2^- by using a lucigenin-dependent chemiluminescence technique; the removal of the endothelium from aortic rings obtained from rabbits had no significant effect on basal O_2^- level. Using the same technique, Ohara, Peterson & Harrison (1993) even observed an increase in basal O_2^- production in isolated rabbit aorta after the mechanical removal of the endothelium.

The discrepancy between these studies is not yet clear. The answer, however, may be important, since it is known that O_2^- rapidly inactivates NO (Moncada & Higgs, 1993). In aqueous solution at pH 7·4, NO and O_2^- react with each other to yield the peroxynitrite anion ($ONOO^-$), which is highly toxic due to its decomposition product, the hydroxyl radical, and is considered to play a role in the process of atherosclerosis (White *et al.* 1994). Furthermore, an excess production of O_2^- has been suggested to be responsible for the impaired endothelial control of vascular tone under pathological conditions, including hypercholesterolaemia (Ohara *et al.* 1993; Mügge *et al.* 1994), hypertension (Nakozono, Watanabe, Matsuno, Sasaki, Sato & Inoue, 1991) and diabetes (Hattori, Kawasaki, Abe & Kanno, 1991). The precise location of excessive O_2^- production, however, has been poorly characterized.

In the present study, we investigated the production and/or release of O_2^- in intact coronary arterial ring preparations and homogenates using a lucigenin-enhanced chemiluminescence technique. In contrast to previous studies using a similar method to detect the vascular release of O_2, the chemiluminescence technique was validated in the present study with an O_2^--generating xanthine/xanthine oxidase system and the experimental conditions for quantifying O_2^- generation were characterized and optimized. Because this technique has been used in the past by several investigators to quantify the vascular release of O_2^-, our intention was to report on the validation and experimental set-up of this techique in greater detail than has been done previously. Furthermore, we utilized histochemical techniques involving the electron acceptor Nitroblue Tetrazolium (NBT) in order to determine the location of O_2^- production. Our results suggest that the endothelium in intact arterial rings does produce O_2^- under basal conditions.

METHODS

Coronary arteries

Pig hearts were freshly obtained from the abbattoir. Immediately after the hearts had been removed they were rinsed and transported in ice-cold Krebs bicarbonate buffer. The left anterior descending coronary arteries were dissected free of adjacent tissue, taking care not to damage the endothelium. Arteries were cut into 3 mm-long ring segments. The rings were kept in oxygenated Krebs bicarbonate buffer at 37 °C for 120 min before use. The Krebs bicarbonate buffer had the following composition (mM): NaCl, 118·3; KCl, 4·7; $CaCl_2$, 1·8; $MgSO_4$, 1·2; KH_2PO_4, 1·2; $NaHCO_3$, 25; Na-EDTA, 0·026; glucose, 11·1; pH 7·40 aerated with 95% O_2–5% CO_2.

Measurement of O_2^-

The generation of O_2^- was measured using a lucigenin (bis-*N*-methylacridinium nitrate)-enhanced chemiluminescence (CL) technique. The light reaction between O_2^- and lucigenin (Brandes *et al.* 1994) was detected in a scintillation counter with six photomultiplier tubes (Biolumat LB 9505; Berthold, Wildbad, Germany). The final concentration of lucigenin used was 0·23 mM dissolved in either Krebs bicarbonate, phosphate or Krebs–Hepes (10 mM) buffer. The phosphate buffer had the following composition: Na-EDTA, 0·025 mM; KH_2PO_4, 0·1 M; and Na_2HPO_4, 0·1 M; and was titrated to pH 7·40. The Krebs–Hepes buffer had the following composition (mM): Hepes acid, 10·0; NaCl, 135·3; KCl, 4·7; $CaCl_2$, 1·8; $MgSO_4$, 1·2; KH_2PO_4, 1·2; Na-EDTA, 0·026; glucose, 11·1; pH 7·40. The buffers used to dissolve lucigenin were not aerated with carbogen. The 3 ml scintillation vials were made of polystyrene; the total volume of lucigenin–buffer solution was 600 μl. After the background CL activity had been measured for 5 min, the ring preparations were carefully positioned in the test vials, and after 5–10 min of equilibration, photon emission was continuously recorded for 5 min. The specific CL signal was expressed as counts per minute (c.p.m.) minus the average background activity. The reaction mixture (lucigenin dissolved in buffer) without arterial rings did not generate CL signals above the background activity when being observed for ≤ 60 min periods. After measurement of the CL signals, the aortic rings were blotted, dried and weighed.

In some experiments, aortic rings were exposed for 30 min to Krebs bicarbonate buffer containing either 5 mM sodium diethyldithiocarbamate (DETC) in order to inhibit vascular Cu–Zn SOD activity (Mügge, Elwell, Peterson & Harrison, 1991*a*), 10 μM indomethacin to inhibit vascular cyclo-oxygenase activity, or 1 mM oxypurinol to inhibit xanthine oxidase (XO) activity. In other experiments, rings were exposed for 30 min to Krebs bicarbonate buffer containing either 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) in order to inhibit endothelial NO synthase (Moncada & Higgs, 1993), or were pre-incubated with 1 mM potassium cyanide (KCN) for 15 min to inhibit the mitochondrial respiratory chain reaction. Other ring preparations were incubated for 6 h in aerated Krebs bicarbonate buffer containing 10 mM 2,4-diamino-6-hydroxypyrimidine (DAHP) for the inhibition of tetrahydrobiopterin

synthesis (Cosentino & Katusic, 1995). After incubation, rings were washed ⩾ 3 times in Krebs buffer and placed immediately into the scintillation counter.

The addition of xanthine (X; 1–25 nmol per vial) and xanthine oxidase (2 mU per vial) to lucigenin produced dose-dependent transient CL signals. In order to determine the degree of O_2 reduction by XO under our experimental conditions, the reduction of 80 μM ferricytochrome *c* (adsorbance at 550 nm; extinction coefficient, $2{\cdot}11 \times 10^4$ l mol^{-1} cm^{-1}) was spectrophotometrically determined in the presence of 2 μg ml^{-1} catalase, and the amount of O_2^- produced from each mole of X was calculated. In our assay, the yield of O_2^- from each mole of X was 40·8 ± 1·4% ($n = 8$). This value was used in order to calculate the amount of O_2^- equivalent to the lucigenin CL signal. Experiments were performed in the absence and presence of oxypurinol (0·1–1000 μM), ascorbic acid (0·1–1000 μM), bovine Cu–Zn SOD (0·001–100 U ml^{-1}) and 4,5 dihydroxy-1,3-benzene disulphonic acid salt (tiron; 0·1 μM to 100 mM).

Measurement of O_2^- in homogenates

Coronary arteries (approximately 200 mg of tissue) were placed in 1 ml of ice-cold (4 °C) Krebs–Hepes buffer, cut with a pair of scissors and homogenized using a pre-cooled Ultra-Turrax homogenizer (3 × 10 s bursts). The crude homogenate was incubated for 30 min at 37 °C in Krebs–Hepes buffer containing 1000 mg l^{-1} collagenase, 125 mg l^{-1} elastase, 1000 mg l^{-1} aprotinin and 250 mg l^{-1} trypsin inhibitor. Crude homogenate was washed twice with Krebs–Hepes buffer to remove collagenase (centrifuged at 500 *g* for 10 min). The pellet was resuspended in 1 ml Krebs–Hepes buffer with a motor-driven glass–Teflon homogenizer (3 × 10 s), and centrifuged at 12000 *g* for 20 min in order to remove mitochondria, after which the supernatant was centrifuged at 100000 *g* for 60 min. The final pellet was then resuspended in phosphate–EGTA buffer (mM: 50 phosphate buffer (pH 7·0); 1 EGTA and 100 sucrose). In the chemiluminescence experiments, 50 μl of either resuspended pellet or supernatant was added to 390 μl phosphate–EGTA buffer with 10 μl lucigenin (0·23 mM) in the presence or absence of 50 μl NADH or NADPH (100 μM). The protein content was measured according to the method of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of total SOD activity

SOD activity was determined in a cell-free O_2^--generating X/XO assay (33·8 μM X, 0·334 mU ml^{-1} XO; pH 9·0; 37 °C) by lucigenin-enhanced CL (250 μM lucigenin dissolved in Tris buffer, 1 mM EDTA). SOD activity was calibrated with commercially available bovine Cu–Zn SOD from Sigma. This technique is based on the definition of SOD activity by McCord & Fridovich (1968) and has been described previously (Corbisier, Houbion & Remacle, 1987).

NBT histochemistry

Arterial rings (with and without intact endothelium) were incubated for 60–180 min in Krebs bicarbonate buffer (37 °C, gassed with 95% O_2–5% CO_2) containing 1 mg ml^{-1} NBT. Some rings were incubated together with 150 U ml^{-1} Cu–Zn SOD, 10 mM tiron, or 5 mM DETC. The soluble yellow form of NBT was reduced by superoxide anions to form blue formazan as an insoluble precipitate in tissues (Nineham, 1954). After incubation, the rings were washed in saline (0·9% NaCl), placed in 3·5% formaldehyde (v/v), and kept overnight in the refrigerator (4 °C). Specimens were either embedded in paraffin or placed in OCT Tissue Tek embedding medium (Miles Inc., Elkhardt, IN, USA) and frozen in liquid N_2-cooled isopentane. The frozen specimens were cut by cryostat into 10 or 20 μm sections, mounted onto poly-L-lysine-coated glass slides and counterstained with either Eosine or Licht-Green. They were immediately investigated by light microscopy, and photographed using a Kodak Ektachrome T64 film. In open cut arterial rings, the luminal surface was digitized using a scanner (HPIIIc, Hewlett Packard) with identical pre-settings for brightness, contrast, intensity and colours. The average brightness of each ring was determined on an arbitrary scale ranging from 0 (black) to 255 (white). The brightness of ring preparations incubated with NBT was set at 100%. Changes in the amount of formazan formation by SOD, tiron or DETC were expressed as different degrees of brightness compared with NBT-incubated control ring preparations.

Confirmation of endothelial cell removal

For some arterial ring preparations, the successful removal of endothelium was confirmed in organ bath experiments measuring isometric tension as described previously (Mügge, Elwell, Peterson, Hofmeyer, Heistad & Harrison, 1991*b*). The coronary arteries were preconstricted with prostaglandin $F_{2\alpha}$ (3 μM); after reaching a stable contraction plateau, maximal concentrations of the endothelium-dependent dilators bradykinin (0·1 μM) and A23187 (calcium ionophore; 10 μM) were added. In vessels with intact endothelium, bradykinin or A23187 induced almost 100% relaxations; in contrast, relaxations were completely abolished in denuded arteries. Denuded arteries could still be relaxed by adding an endothelium-independent dilator nitroglycerine (1 μM). The ring preparations, used for organ bath studies, were not used for further chemiluminescence experiments. The complete removal of the endothelium was also confirmed in sample preparations by *en face* light microscopy after silver staining.

Drugs

L-NAME, indomethacin, tiron, phorbol 12-myristate 13-acetate (PMA), lucigenin, DETC, NBT, DAHP, oxypurinol, X, XO, SOD, the reduced form of β-nicotinamide–adenine dinucleotide (NADH), the reduced form of β-nicotinamide–adenine dinucleotide phosphate (NADPH) and Hepes free acid were purchased from Sigma (Deisenhofen, Germany). Diphenyliodonium (DPI) was from Aldrich (Deisenhofen, Germany). Oxypurinol and X were dissolved in 0·01 N KOH, XO stock solutions (1 U ml^{-1}) were prepared in 2·3 M ammonium sulphate. Indomethacin was dissolved in 20 mM Na_2CO_3, adjusted with 0·1 N HCl to pH 7·4. PMA stock solutions were dissolved in dimethylsulphoxide (DMSO). Other stock solutions were freshly prepared in distilled water.

Statistics

Values are means ± S.E.M. and statistical analysis was carried out using Student's *t* tests for paired or unpaired observations or the Wilcoxon signed rank test. A value of $P < 0{\cdot}05$ was considered as significant.

RESULTS

Cell-free O_2^--generating assay

In a cell-free xanthine/xanthine oxidase (X/XO) assay, the magnitude of the CL signal was dependent on the concentration of X and XO used. Figure 1 shows an example of a CL signal generated by increasing concentrations of X (1–16 nmol) in the presence of one XO concentration (2 mU ml^{-1}). Figure 2*A* shows the CL signal in response to increasing concentrations of XO (0·1–4 mU ml^{-1}) in the presence of one X concentration (500 μM). In Fig. 2*B*, corresponding changes in a ferricytochrome *c* assay are shown. Whereas the reduction of ferricytochrome *c* by

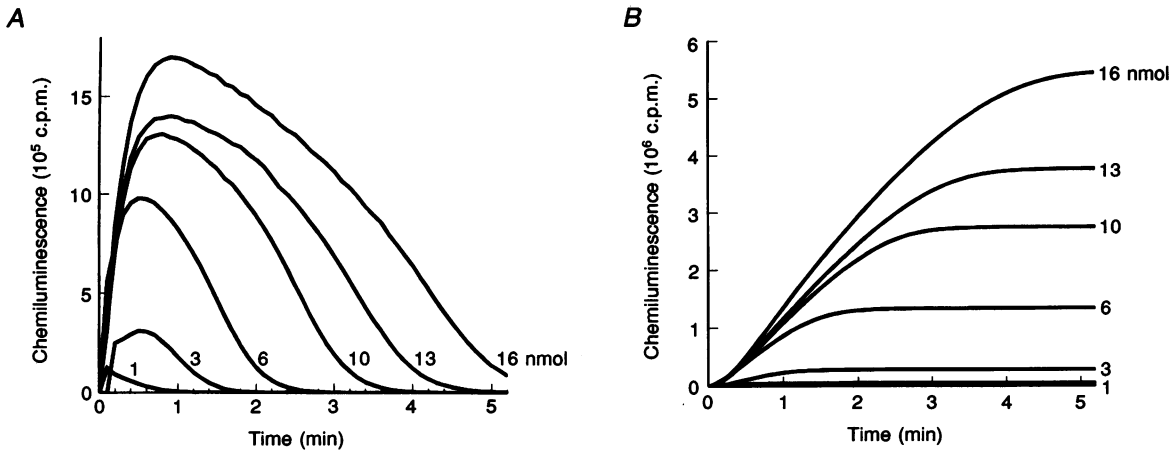

Figure 1. Generation of O_2^- by a cell-free X/XO system

Measurement of radicals by lucigenin-enhanced CL (expressed in c.p.m.). Increasing concentrations of X (1–16 nmol) were incubated with 2 mU ml^{-1} XO. A representative example out of 5 experiments is shown; *A* shows the transient CL signal, *B* shows the integral of the CL signal.

X/XO was almost linear, the CL signal was curvilinear: the sensitivity of lucigenin to generate photon emission in response to X/XO was lower at the low concentration range of XO (< 1 mU ml^{-1}, corresponding to < 100 000 c.p.m. in the CL) compared with higher concentrations of XO. The regression equation for the low concentration range was $O_2^- = CL \times 7{\cdot}8$ (units for O_2^- and CL are fmol min^{-1} and c.p.m., respectively).

The CL signal generated by 0·6 mU ml^{-1} XO and 500 μM X (21 800 ± 1500 c.p.m.) could be completely blocked by adding the XO inhibitor oxypurinol, the radical scavengers ascorbic acid and tiron, and bovine Cu–Zn SOD. The concentrations of oxypurinol, ascorbic acid, tiron and SOD, which inhibited the CL response generated by X/XO to 50%, were 7·9 ± 0·8 μM, 8·4 ± 1·0 μM, 58·8 ± 11·0 μM and 0·163 ± 0·02 U ml^{-1}, respectively (n = 4–5 experiments for each inhibitor).

Various drugs were screened for unspecific O_2^- scavenger properties in this X/XO cell-free system (0·6 mU ml^{-1} XO, 500 μM X). Table 1 summarizes changes in the CL signal in response to these drugs. Several drugs tested showed a significant interference with the CL signal.

Basal CL signal in response to coronary artery rings

The addition of intact rings to vials containing lucigenin produced a significant CL signal above background activity. Depending on the buffer used to dissolve lucigenin, different courses of the CL signal were noted. Using Krebs bicarbonate buffer, a significant increase in the CL signal with a plateau between 12 and 15 min after addition of vessels was observed. The pH of the lucigenin–Krebs

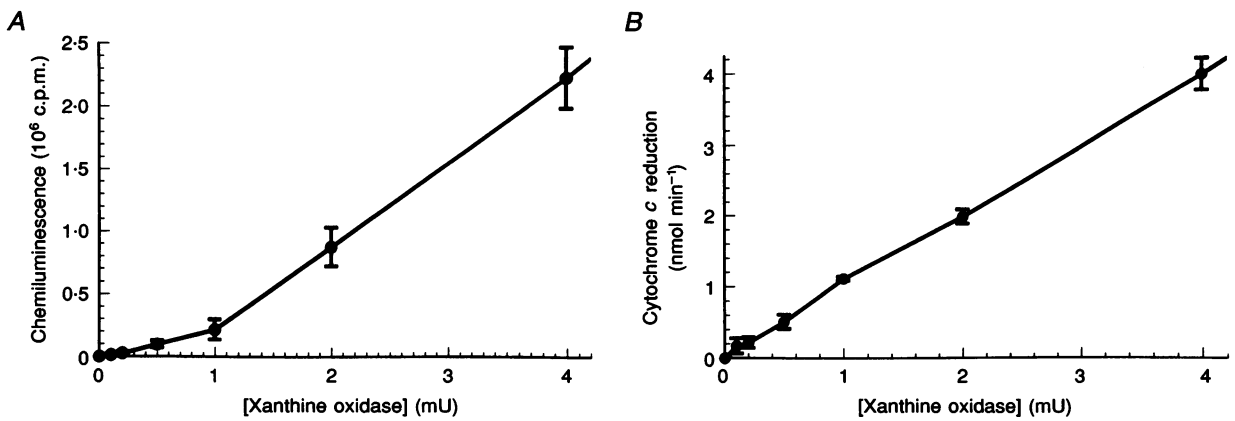

Figure 2. Generation of O_2^- by a cell-free X/XO system

Measurement of radicals by lucigenin-enhanced CL (*A*) and by reduction of 80 μM ferricytochrome *c* (*B*). Increasing concentrations of XO (0·1–4 mU ml^{-1}) were incubated with 500 μM X. Values are means ± S.E.M., n = 5 experiments each. Note that the generation of O_2^- by X/XO as measured by the cytochrome *c* assay showed a linear relationship, whereas the CL signal showed a curvilinear relationship with less sensitivity for the low concentration range of XO (< 1 mU ml^{-1}).

Table 1. Interference of various drugs with the CL signal of a cell-free O_2^--generating system

Drugs (concentration)	Change in CL signal (%)	n	P
Control (10 μl bidest)	<5	5	n.s.
Acetylcholine (1 μM)	<5	5	n.s.
Bradykinin (0·1 μM)	<5	5	n.s.
DAHP (10 mM)	<5	5	n.s.
DETC (5 mM)	−66	5	<0·05
DPI (10 μM)	−16·6	4	<0·05
DMSO (1 %)	<5	4	n.s.
KCN (1 mM)	>10000	5	<0·05
NADH (100 μM)	<5	5	n.s.
NADPH (100 μM)	<5	5	n.s.
L-NAME (100 μM)	<5	5	n.s.
L-NMMA (100 μM)	+17	5	<0·05
L-NOA (100 μM)	< 5	5	n.s.
Rotenon (1 mM)	>10000	5	<0·05
Serotonin (10 μM)	−35	5	<0·05
Substance P (0·1 μM)	<5	5	n.s.
Quinacrine (1 mM)	−95	4	<0·05

n, number of experiments; L-NMMA, monomethy-L-arginine; L-NOA, nitro-L-arginine.

bicarbonate buffer increased after the addition of the vessels on average from 7·40 to 7·58 ($n = 4$, Fig. 3*A*). Using Krebs–Hepes buffer or phosphate buffer, a short decline of the CL signal was followed by a sustained plateau of the signal over a period of at least 15 min (Fig. 3*A*; data shown for Krebs–Hepes buffer). Measurements of the pH of the lucigenin–Krebs–Hepes buffer (or phosphate buffer) before and after addition of the vessels revealed a stable value of 7·40 ($n = 4$).

The effect of pH on the CL signal in the presence of arterial rings is shown in Fig. 3*B*. For this subset of experiments, lucigenin was dissolved in a phosphate buffer in order to adjust this buffer with NaOH or HCl to different pH values

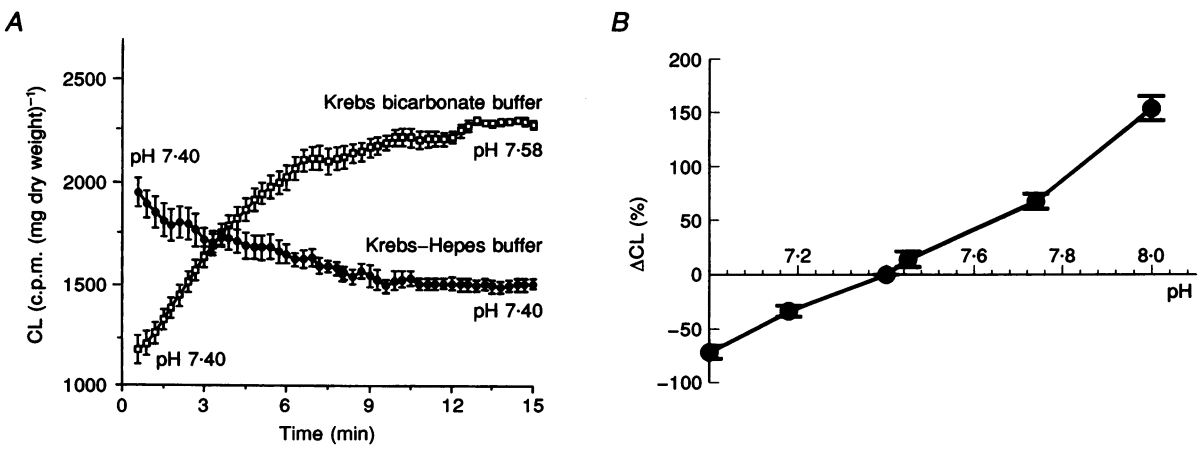

Figure 3. Influence of pH on the CL signal in response to pig coronary rings

A, CL signals in response to intact pig coronary rings. Lucigenin (0·23 mM) was dissolved in either Krebs bicarbonate or Krebs–Hepes buffer. The pH of the lucigenin–buffer solution was measured immediately after addition of arterial rings, and at the end of the experiment at 15 min. CL signal is expressed as c.p.m. (mg dry weight)$^{-1}$ after the subtraction of background activity. Values are means ± S.E.M., $n = 4$ experiments for each buffer. *B*, influence of pH on the CL signal in response to intact pig coronary artery rings. Lucigenin was dissolved in phosphate buffer. Phosphate buffer was adjusted by adding NaOH or HCl to various pH values ranging from 7·00 to 8·00. The CL response at pH 7·4 was set as baseline, the CL signal at various pH values were expressed as the percentage change compared with baseline. Values are means ± S.E.M., $n = 4$ experiments for each pH value.

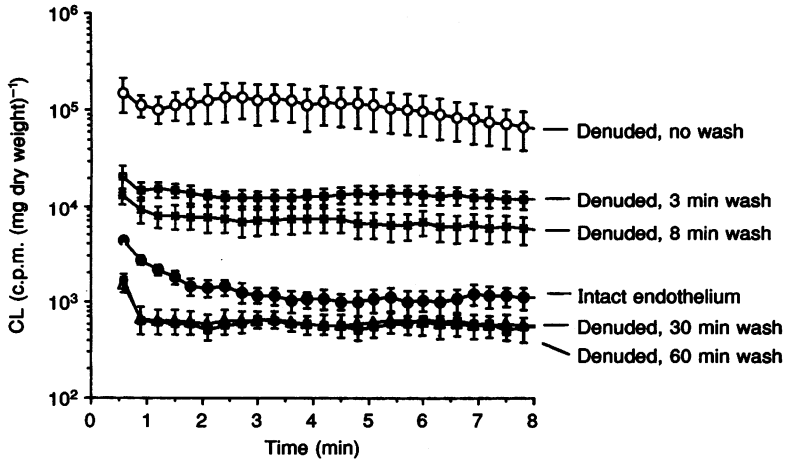

Figure 4. Influence of endothelium on the CL signal in response to pig coronary arteries

Arteries were mechanically denuded and placed into the scintillation counter either immediately after the denudation procedure or after different washing periods (3–60 min) in Krebs buffer. The CL signal in response to denuded arteries was compared with that obtained from arteries with intact endothelium. The curves for the intact arteries (with different washing periods) were identical, therefore, only one curve is shown. The CL signal is expressed as c.p.m. (mg dry weight)$^{-1}$. Values are means ± S.E.M., number of experiments is 5.

(7·00–8·00). Depending on actual pH, the CL signal substantially varied in response to arterial rings.

Because the CL signal was dependent on the actual pH, care was taken to adjust it to 7·40. In the following experiments, we preferred to measure vascular generation of O_2^- in Krebs–Hepes buffer. Under these conditions, coronary artery ring preparations from pigs generated on average 1157 ± 106 c.p.m. (mg dry weight)$^{-1}$ ($n = 16$), which corresponded to an O_2^- generation of 9·0 ± 0·8 pmol O_2^- min^{-1} (mg dry weight)$^{-1}$.

Effect of denudation on basal CL signal

Arterial rings were mechanically denuded by carefully rubbing the luminal surface with a wooden applicator. The complete removal of endothelium was confirmed in some randomly chosen rings ($n = 10$) by organ bath studies or by histological examinations (see Methods; data not shown). To evaluate the influence of endothelial removal, denuded arteries or, for comparison, arteries with intact endothelium were washed in Krebs bicarbonate buffer (37 °C, gassed with 95% O_2–5% CO_2 before use) at various time intervals (3–60 min) before they were added into the scintillation counter. In this set of experiments, intact arteries produced a stable CL signal of approximately 1100 c.p.m. (mg dry weight)$^{-1}$ for at least 8 min, irrespective of the time interval at which they were stored before use. Denuded arteries which were immediately placed into the scintillation counter without a wash in Krebs buffer, produced a CL signal in the range of 100 000 c.p.m. (mg dry weight)$^{-1}$. Increasing the time intervals for washing, the CL signals in response to denuded arteries decreased. Denuded arteries, which were washed for 30 and 60 min before they were placed into the scintillation counter, produced a CL signal which was consistently lower than that obtained with intact arteries (Fig. 4). The CL signals in response to intact and denuded arteries (60 min wash) were 1157 ± 106 and 880 ± 80 c.p.m. (mg dry weight)$^{-1}$, respectively ($P < 0·05$; $n = 16$); these values correspond to 9·0 ± 0·8 and 6·8 ± 0·6 pmol O_2^- min^{-1} mg^{-1}, respectively. Thus, endothelium may account on average for 24% of the total CL signal from the vessel wall.

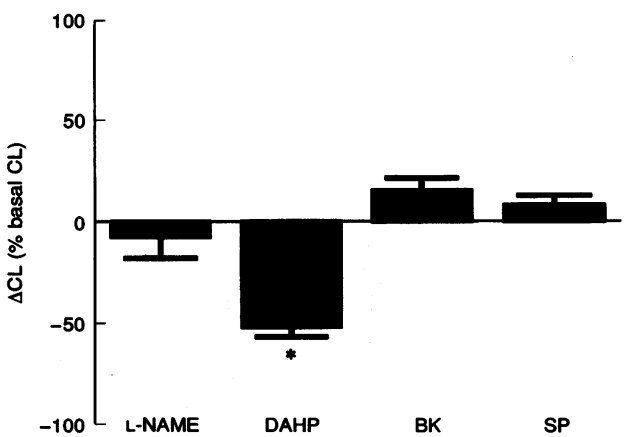

Figure 5. Influence of inhibitors and stimulators of NOS on the CL signal in intact pig coronary arteries

Rings were incubated in Krebs bicarbonate buffer containing either 100 μM L-NAME (30 min), or 10 mM DAHP (6 h). Bradykinin (BK; 0·1 μM) or substance P (SP; 0·1 μM) were added to other rings. CL signals are expressed as the percentage change compared with controls. Values are means ± S.E.M., $n = 10$–12 for each inhibitor; * $P < 0·05$ *versus* control.

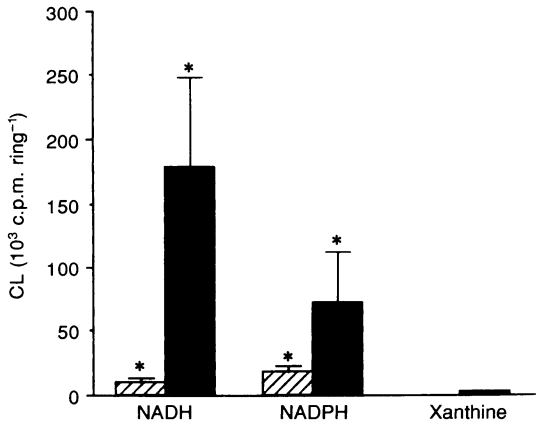

Figure 6. Generation of O_2^- by intact pig coronary artery rings following addition of NADH, NADPH and xanthine
▨, 10 μM drug; ■, 100 μM drug. O_2^- production is expressed in c.p.m. per vessel ring. Values are means ± S.E.M., $n = 4$; * $P < 0{\cdot}05$ *versus* baseline values.

Inhibition and stimulation of CL signal in intact pig coronary arteries

The non-enzymatic scavenger tiron (0·001–10 mM), Cu–Zn SOD (0·001–100 U ml^{-1}) and DPI (0·01–1000 μM), an inhibitor of NAD(P)H-utilizing flavoproteins (O'Donnell, Tew, Jones & England, 1993), inhibited the CL signal in response to arterial rings with intact endothelium. Tiron and DPI strongly inhibited the CL signal (⩾ 85%), SOD was partially effective (maximal inhibition, 53 ± 8%). The IC_{50} values for tiron, DPI and SOD were 0·23 ± 0·07 mM, 1·85 ± 5·33 μM and 1·13 ± 1·38 U ml^{-1}, respectively ($n = 4$–6 experiments for each inhibitor). In contrast to tiron, DPI or SOD, pre-incubation of arterial rings with oxypurinol (1 mM) or indomethacin (10 μM) for 30 min had no significant effect on the CL signal ($n = 5$). These results suggest that both XO and cyclo-oxygenase have only minor if any influence at all on the vascular O_2^- generation, at least under basal conditions in intact ring preparations.

Figure 5 demonstrates the influence of stimulation and inhibition of NOS on the CL signal in response to arterial rings with intact endothelium. Arterial rings were incubated for 30 min in Krebs bicarbonate buffer containing 100 μM L-NAME. Other rings were pre-incubated for 6 h in Krebs bicarbonate buffer containing 10 mM DAHP, an inhibitor of the GTP cyclohydrolase I (tetrahydrobiopterin biosynthesis). The CL signals were compared with those generated by intact arterial rings, which were incubated for identical time periods in control buffer. Pretreatment of rings with DAHP significantly decreased the CL signal by about 55%; in contrast, pretreatment with L-NAME had only a minor effect on the CL signal (−7·4%, P = n.s.). The addition of either bradykinin (0·1 μM) or substance P (0·1 μM) to arterial rings with intact endothelium (in order to stimulate NOS) had no significant effect on the CL signal.

Incubation of arterial rings with KCN (1 mM, 15 min) or DETC (5 mM, 30 min) increased the CL signal by 48 ± 15 and 184 ± 55%, respectively ($n = 10$). The removal of endothelium had no effect on this increase of CL (data not shown), and the DETC-induced increase in CL could be reduced by 46 ± 9% ($n = 19$) by adding 150 U ml^{-1} Cu–Zn SOD.

The addition of 5 μM PMA to intact arterial rings (in order to stimulate protein kinase C) increased the CL signal by 136 ± 20% ($n = 19$). This increase was abolished in denuded arteries ($n = 19$; data not shown).

The addition of either NADH or NADPH, but not of X (10 and 100 μM), to intact arterial rings produced an excessive generation of O_2^- (Fig. 6). For the higher concentration (100 μM), this increase in CL signal was significantly more pronounced in response to NADH than NADPH (182 000 ± 67 500 *versus* 70 500 ± 46 100 c.p.m. per vessel ring, $n = 4$; $P < 0{\cdot}05$).

O_2^- generation in vessel homogenates

The total SOD activity in the cytosolic fraction of arterial ring homogenates was 1·38 ± 0·28 U (mg protein)$^{-1}$ ($n = 3$),

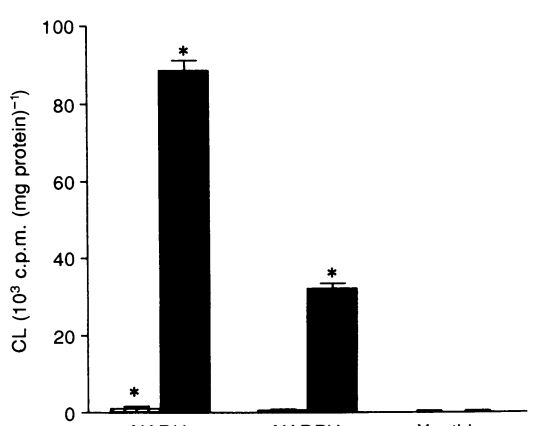

Figure 7. Measurement of O_2^- generation in the particulate and cytosolic fraction of arterial ring homogenates
▨, soluble fraction; ■, membrane fraction. Generation of O_2^- was studied in the presence and absence of NADH, NADPH or xanthine (100 μM), and measured by lucigenin-enhanced chemiluminescence. CL signal is expressed as c.p.m. (mg protein)$^{-1}$. Values are means ± S.E.M., $n = 3$ experiments. Note that NADH and NADPH stimulated O_2^- generation only in the particulate fraction.

and the total SOD activity in the membrane fraction was about 25 times lower ($0{\cdot}055 \pm 0{\cdot}01$ U (mg protein)$^{-1}$; $n = 3$). The addition of either 100 μM NADH or NADPH, but not of 100 μM X, produced an excessive generation of O_2^- in the membrane fraction of the homogenate (Fig. 7). This increase in O_2^- was about 3 times higher in response to NADH than to NADPH ($0{\cdot}68 \pm 0{\cdot}03$ *versus* $0{\cdot}26 \pm 0{\cdot}01$ nmol O_2^- min^{-1} (mg protein)$^{-1}$). In contrast to the membrane fraction, almost no CL signal in response to NADH or NADPH was observed in the cytosolic fraction (< 0·01 nmol O_2^- min^{-1} (mg protein)$^{-1}$); again, the addition of X did not increase the CL signal.

Reduction of NBT in pig coronary arteries

In order to test the hypothesis that the endothelium may be a source of O_2^- generation, arterial rings with and without intact endothelium were incubated with NBT. Figure 8*A* shows a representative macroscopic view of the luminal side of two arterial ring preparations (out of a series of 30 individual experiments); an intense blue colouring of the

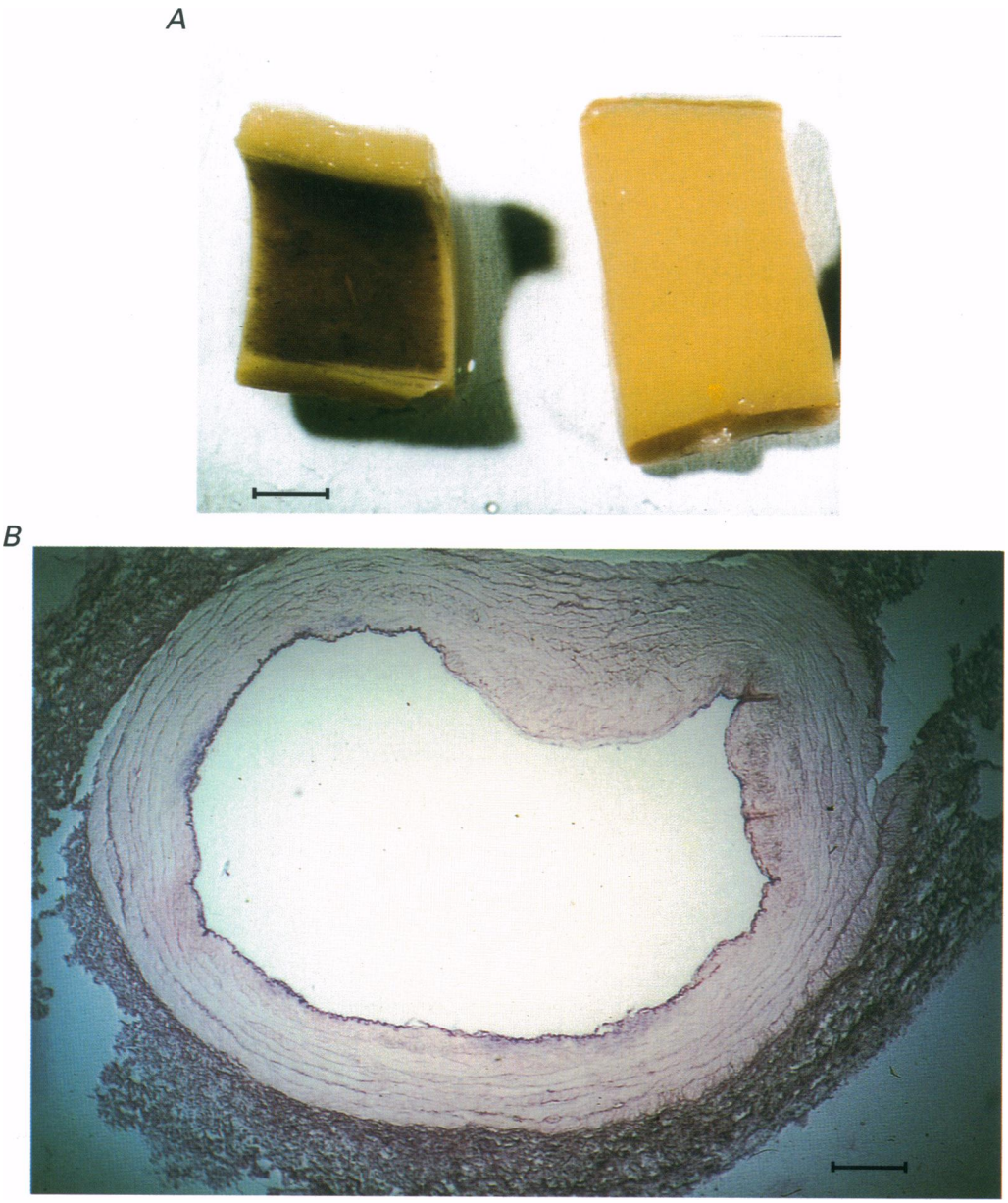

Figure 8. Reduction of NBT (formazan formation) in pig coronary arteries

A, incubation of two pig coronary artery rings (left side with, right side without intact endothelium) with 1 mg ml^{-1} NBT for 120 min. The luminal surface is shown. Note that the arterial ring with intact endothelium, but not the denuded vessel, showed a positive staining with blue formazan; scale bar, 1 mm. *B*, incubation of a pig coronary artery ring with 1 mg ml^{-1} NBT for 180 min. A cross-section of a specimen is shown embedded in paraffin and counterstained with Eosin for 10 s; scale bar, 300 μm. Note that staining with blue formazan occurred predominantly in the intima and adventitia.

luminal surface was only observed in those rings with an intact endothelium, but not in the denuded arteries. Figure 8*B* shows a cross-section through an arterial ring with intact endothelium. Whereas the media showed minor blue staining, the intima (and the adventitia) showed an intense blue colouring due to NBT reduction. The addition of 150 U ml^{-1} Cu–Zn SOD or 10 mM tiron significantly increased the brightness (intensity of blue colouring due to formazan) of the luminal surface on an arbitrary scale by 31 ± 3 and 45 ± 4% ($n = 5$ for each inhibitor), respectively. In contrast, inhibition of vascular Cu–Zn SOD with 5 mM DETC significantly reduced the brightness of the intimal surface by 40 ± 5% ($n = 6$) compared with control ring preparations.

DISCUSSION

In the present study, two techniques were used for the detection of vascular O_2^- generation: lucigenin-enhanced chemiluminescence and reduction of NBT. The first technique is assumed to be specific for the detection of O_2^- (for references see Brandes, Dwenger & Mügge, 1994). The CL technique has previously been used by several investigators to quantify vascular generation of O_2^- (Ohara *et al.* 1993; Pagano *et al.* 1993). In the present study, the CL technique was validated in a cell-free X/XO system generating O_2^-, and compared with a cytochrome *c* assay as an alternative method to detect O_2^-. Furthermore, extensive work was carried out to optimize the CL technique for the use of quantifying vascular O_2^- generation.

In contrast to previous reports (Ohara *et al.* 1993; Pagano *et al.* 1993), we observed a curvilinear relationship between the amount of generated O_2^- by X/XO and the CL signal with less sensitivity for the lower concentration range (< 1 nmol O_2^- min^{-1}). This non-linear relationship has to be taken into account if the CL signal is converted to absolute O_2^- values. In comparison to lucigenin, the reduction of cytochrome *c* showed a linear relationship over a wide range of concentrations of O_2^- generated by X/XO. Nevertheless, we preferred the CL techniques, since this method is more sensitive, and probably more specific than the cytochrome *c* assay when O_2^- is measured in vascular tissues. It has been shown previously that haemoproteins such as cytochrome *c* do interfere with NO (Kanner, Harel & Granit, 1991) and can be directly reduced by NOS (Klatt, Schmidt, Uray & Mayer, 1993). Concerning optimizing CL measurements, we observed that the CL signal is strongly dependent on changes in the actual pH of the lucigenin–buffer solution and on the type of buffer used. This has not been reported previously. The most stable pH and CL signals were obtained when vascular tissues were placed into a lucigenin phosphate or Krebs–Hepes buffer. In addition, it was noted that several substances used in subsequent experiments had either scavenger properties or interacted with the background photon emission by lucigenin (see Table 1). Substances that by themselves showed a significant influence on the CL signal were not directly used in the CL assay; instead, vascular rings were pre-incubated with these substances, and great care was taken to wash the arterial rings several times before they were placed into the scintillation counter.

The NBT–histochemical technique is based on the conversion of a soluble salt into an insoluble, blue formazan by using a reducing agent such as O_2^- (Nineham, 1954). However, in contrast to lucigenin, NBT may also be reduced by other electron-donating reactions such as those created by NOS activity (NADPH–diaphorase reaction) (Hope, Michael, Knigge & Vincent, 1991). Thus, particularly in vascular tissue, positive staining with formazan may not only indicate the presence of O_2^- but may also be a marker for NOS activity. The presence of O_2^- scavengers such as tiron and SOD partially reduced, whereas inhibition of vascular SOD activity by DETC significantly enhanced, formazan staining. These observations suggest that formazan staining is partially due to O_2^- generation, and not to NOS activity alone. Since the vessel rings were exposed to carbogen during NBT staining (NBT solution was gassed with carbogen), the NBT staining in the endothelium may be greater than it would generally have been under more physiological conditions.

The purpose of the present study was to test whether intact arterial ring preparations generate O_2^-. Intact pig coronary arteries generate O_2^- in small quantities under basal conditions, which we calculated to be approximately 9 pmol O_2^- min^{-1} (mg dry weight)$^{-1}$. This amount of O_2^- generated by pig coronary arteries is more than 50 times less than that reported previously. Ohara and co-workers (1993) reported a rate of O_2^- generation of about 0·52 nmol min^{-1} (mg dry weight)$^{-1}$ for isolated rabbit aorta. This discrepancy may be due to differences in species, different types of vascular tissue (aorta *versus* coronary artery), but perhaps also due to differences in the standard curve relating to CL signal and absolute O_2^- values, and the use of pH-unstable buffers.

A major result of the present study is that the endothelium may account for about 24% of the total CL signal of the vessel wall. Thus, the endothelial cell layer appears to be a significant source of O_2^- generation of the intact pig coronary artery. This view is supported by the NBT-staining technique which demonstrated formazan formation within the luminal surface of intact arterial ring preparations that disappeared after mechanical denudation. Our findings support previous studies demonstrating O_2^- generation in cultured endothelial cells (Rosen & Freeman, 1984; Matsubara & Ziff 1986*a*,*b*; Holland *et al.* 1990; Mohazzab, Kaminski & Wolin, 1994; Pritchard *et al.* 1995). Our results are in contrast to those of others which failed to demonstrate an endothelial release of O_2^- in isolated aorta obtained from normal rabbits (Ohara *et al.* 1993; Pagano *et al.* 1993). The reason for this discrepancy is not clear; however, we noted that the magnitude of the CL signal was strongly dependent on the time interval during which freshly denuded arteries were washed in Krebs buffer before they were placed into the scintillation counter (see Fig. 4).

The CL signal was greatly enhanced shortly after denudation and declined consistently to the lower levels of the intact vessels after washing intervals > 30 min. Thus, the discrepant results may be due to experimental conditions, i.e. unequal washing time intervals after injury of arterial rings.

The mechanism of O_2^- generation within the arterial wall and in particular from endothelial cells is not known. The vascular release of O_2^- was not influenced by oxypurinol and indomethacin suggesting that neither XO nor cyclooxygenase contributes to O_2^- generation. The non-enzymatic scavenger tiron almost inhibited the CL signal in response to intact coronary arteries, whereas the addition of SOD only partially inhibited the CL signal by about 50%. This difference may be explained by the fact that the macromolecule SOD is not able to penetrate cell membranes (Beckman, Minor, White, Repine, Rosen & Freeman, 1988), whereas small molecules like lucigenin (Paky, Michael, Burke-Wolin, Wolin & Gurtner, 1993) or tiron (Devlin, Lin, Perper & Dougherty, 1981) are. The observation that DPI significantly reduced the vascular generation of O_2^- suggests that a flavoprotein is involved. Endothelial cells are rich in flavoproteins; among others NOS is a calcium–calmodulin-dependent flavoprotein (Moncada & Higgs, 1993). It is possible that NOS is a source not only for NO but also for O_2^-. Accordingly, it has been shown previously that NOS generates O_2^- under certain conditions, such as a lack of substrates (Pou, Pou, Bredt, Snyder & Rosen, 1992) or the presence of native low-density lipoproteins (Pritchard *et al.* 1995). Whether this mechanism of O_2^- generation also occurs in 'normal' intact arterial tissue is not known. The endothelial NOS activity, however, may also indirectly influence O_2^- generation. O_2^- is known to rapidly react with NO in solutions at a rate constant, k, of $6{\cdot}7 \times 10^9$ M^{-1} s^{-1} (Huie & Padmaja, 1993) which is comparable to the rate constant of SOD-catalysed dismutation ($> 10^9$ M^{-1} s^{-1}) (Fielden *et al.* 1974). Thus, the amount of vascular O_2^- generation may be 'regulated' by the intracellular SOD activity and the intracellular level of synthesized NO. In the present study, stimulation or inhibition of NOS had no significant influence on the CL signal. In contrast, inhibition of vascular SOD activity greatly enhanced the CL signal. These results suggest that the vascular O_2^- generation is regulated by SOD activity but not by the actual NO level. Others have also failed to demonstrate a significant interference of NOS activity and O_2^- generation (Pagano *et al.* 1993; Mohazzab *et al.* 1994).

It has been recently suggested that endothelial cells contain NAD(P)H oxidases which could be a major source of O_2^- generation (Mohazzab *et al.* 1994). This suggestion is based on the observation that NADH (and to a lesser extent NADPH) stimulated O_2^- generation in homogenates from cultured bovine coronary artery endothelial cells, which could be impaired by SOD, tiron and DPI. The results of the present study support the findings of Mohazzab *et al.* (1994), we also observed that exogenous NADH (and to a lesser extent NADPH) stimulated the generation of O_2^- in intact pig coronary arteries as well as in the membrane fractions of whole tissue homogenates. These results are in line with the hypothesis that membrane-associated NAD(P)H oxidases are present within the vessel wall and are a source of vascular O_2^- generation. Since NAD(P)H oxidases are flavoproteins, this hypothesis may also explain the observation that iodonium compounds such as DPI are effective inhibitors of vascular O_2^- generation. Parts of a vascular NAD(P)H oxidase (a cytochrome b-558 α-subunit) were recently isolated and cloned in rat vascular smooth muscle cells (Fukui, Lassegue, Kai, Alexander & Griendling, 1995).

In order to further characterize the source of vascular O_2^- generation, arterial rings were incubated with KCN, in order to inhibit the mitochondrial respiratory chain reaction, and with the phorbol ester PMA, in order to stimulate protein kinase C. Surprisingly, an increased CL signal after incubation with KCN was observed. This unexpected finding may be due to a potent inhibition of vascular Cu–Zn SOD by KCN. Using the chelator DETC for inhibition of vascular Cu–Zn SOD, an excessive generation of O_2^- was observed. Thus, the KCN-induced generation of O_2^- may be, at least partially, explained by additional inhibitory effects of KCN on vascular SOD activity. The stimulation of protein kinase C with PMA produced an enhanced generation of O_2^-. This increased generation of O_2^- was abolished after denudation of arterial rings. These results suggest that an endothelial protein kinase C-dependent process may contribute to vascular O_2^- generation.

Incubation of intact arterial tissue with DAHP resulted in a significant reduction of vascular O_2^- generation. This incubation protocol has been shown previously to reduce the endogenous level of tetrahydrobiopterin (BH_4) (Kaufman, 1993). BH_4 is a cofactor for endothelial NOS and for a variety of other monoxygenases (Marletta, 1993). Our initial aim to investigate a link between BH_4 and O_2^- was based on the assumption that a lack of cofactor for NOS would result in an inhibition of NO production and may, therefore, enhance O_2^- generation. Meanwhile, it has been shown that BH_4 directly generates O_2^- by autoxidation (Mayer, Klatt, Werner & Schmidt, 1995). Thus, our observation is in agreement with the idea that the reduction of vascular BH_4 synthesis results in a reduced formation of O_2^-.

In summary, isolated pig coronary arteries generate O_2^- at a rate of about 9 pmol min^{-1} (mg dry weight)$^{-1}$ as measured by lucigenin-enhanced chemiluminescence. It appears that the endothelial layer significantly contributes to vascular O_2^- generation. This generation of O_2^- is regulated by intrinsic SOD activity. It is suggested that basal vascular O_2^- generation is mainly due to a membrane-bound NAD(P)H oxidase activity and/or tetrahydrobiopterin-dependent processes.

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