

In situ detection of spontaneous superoxide anion and singlet oxygen production by mitochondria in rat liver and small intestine

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Summary

In the present study, the endogenous formation of reactive oxygen species was localized in rat liver and small intestine. The 3,3'-diaminobenzidine (DAB)-Mn²⁺ technique in which cobalt ions were included in the incubation medium was applied to unfixed cryostat sections of intact tissues. Addition of manganese ions to the DAB-Co²⁺-containing medium greatly increased the amounts of final reaction product formed compared with incubations with only DAB and cobalt ions. In liver, a blue final reaction product was deposited, particularly in hepatocytes surrounding portal tracts. In the small intestine, the DAB-cobalt complex was mainly found at the basal side of enterocytes. Goblet cells remained unstained. Electron microscopical images revealed that an electron-dense reaction product was exclusively present at both inner and outer membranes and at the intermembrane space in mitochondria of liver parenchymal cells and duodenal enterocytes. It was shown that the spontaneous formation of final reaction product was enzymatic and dependent on the presence of oxygen in the medium. Sulphide decreased the reaction, which may indicate that cytochrome *c* oxidase was partially involved. Benzoquinone and histidine, which are scavengers of superoxide anions and singlet oxygen respectively, reduced the amount of final reaction product considerably. Furthermore, the formation of final reaction product was sensitive to specific inhibitors of NADH:coenzyme Q reductase and aldehyde oxidase, indicating that these enzymes were at least partly responsible for the generation of superoxide anions and singlet oxygen and for the formation of the DAB-cobalt complex.

Introduction

During the last decades, it has become clear that reactive oxygen species (ROS), which include superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), singlet oxygen (O₂(¹ΔG)) and hydroxyl radicals (OH·), play an important role in a variety of physiological and pathological processes (for reviews, see Halliwell *et al.*, 1992; Cheeseman & Slater, 1993). Important intracellular sources of ROS are the mitochondrial electron transport chain, peroxisomal oxidases, soluble enzymes, such as xanthine oxidase and aldehyde oxidase, endoplasmic reticulum, nuclear membrane electron transport chain and plasma membrane (Freeman & Crapo, 1982; Kehrer, 1993). The production of ROS by these intracellular sources is of

physiological importance and does not cause damage to surrounding structures under normal conditions. Tissue injury is avoided because levels of ROS are controlled by endogenous antioxidant systems, such as superoxide dismutase, catalase, peroxidase, reduced glutathione, vitamin E and vitamin C (Kehrer, 1993). Increased levels of ROS are thought to play an important role in pathological conditions, such as ischaemia-reperfusion, carcinogenesis, atherosclerosis and ageing (Halliwell *et al.*, 1992; Kontos *et al.*, 1992; Cheeseman & Slater, 1993; Rauen *et al.*, 1994).

Our group is trying to elucidate sources of oxygen radicals in rat liver and small intestine during reperfusion after ischaemia (Frederiks *et al.*, 1993; Kooij *et al.*, 1994; Frederiks & Bosch, 1995). These studies revealed that an important role of the

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conversion of xanthine dehydrogenase to xanthine oxidase in ROS production during reperfusion after ischaemia can be excluded. In accordance with the findings of Babbs *et al.* (1991), we assume that extracellular formation of oxygen radicals may occur and then conversion of xanthine dehydrogenase into xanthine oxidase in the blood may be held responsible. However, mitochondria are proposed as a possible intracellular source of ROS during reperfusion after ischaemia (Gonzalez-Flecha *et al.*, 1993). Therefore, we decided to investigate the exact localization of endogenous formation of ROS in rat liver and small intestine by means of a cytochemical technique based on the principles described for the histochemical localization of ROS production in polymorphonuclear leucocytes (Graham & Karnovsky, 1996; Briggs *et al.*, 1975, 1983, 1986; Karnovsky, 1994). Superoxide anions were demonstrated with the use of 3,3'-diaminobenzidine (DAB) and manganese ions (Mn^{2+}). Mn^{2+} is oxidized to Mn^{3+} by O_2^- and, subsequently, Mn^{3+} oxidizes DAB by polymerization. Recently, this technique was applied by Steinbeck *et al.* (1994) on intact living tissues to investigate the relationship between superoxide anion production and the presence of NADPH oxidase in osteoclasts that actively resorb bone. Cobalt ions were added by us to improve the sensitivity of the procedure (Hsu & Soban, 1982). Moreover, we studied the specificity of the technique and the properties of the system responsible for the formation of the final reaction product.

Materials and methods

Chemicals used

NADP, NADPH, NAD, NADH and superoxide dismutase (SOD; from bovine erythrocytes) were obtained from Boehringer (Mannheim, Germany); cytochrome *c*, histidine, KCN, NaN_3 , rotenone, Triton X-100 and $CoCl_2$ from Merck (Darmstadt, Germany); polyvinyl alcohol (weight average M_r 70 000–100 000), p-benzoquinone, allopurinol, menadione, disulphiram, hypoxanthine, catalase (from bovine liver) and $MnCl_2$ from Sigma (St Louis, MO, USA); 4-hydroxymercuribenzoic acid (PCMB) and 3-amino-1,2,4-triazole from Serva (Heidelberg, Germany); N-ethylmaleimide (NEM) from BDH (Poole, UK); 3,3'-diaminobenzidine tetrahydrochloride (DAB) from Fluka Chemika (Buchs, Switzerland); Agar Noble from DIFCO Laboratories (Detroit, MI, USA); mannitol from Janssen Chimica (Beerse, Belgium).

Animals and tissue processing

Male Wistar rats (TNO, Zeist, The Netherlands) weighing 200–250 g were used. The animals had free access to laboratory diet and tap water and were housed under constant environmental conditions. The animals were killed under ether anaesthesia. Liver and small intestine were removed immediately after sacrifice and small

fragments up to 5 mm thick were frozen in liquid nitrogen and stored at $-80^{\circ}C$ until further use (Van Noorden & Frederiks, 1992).

Histochemical procedures

Cryostat sections (8 μ m thick) were cut on a Bright motor-driven cryostat with automatic speed control to ensure constant section thickness at a cabinet temperature of $-25^{\circ}C$. The sections were picked up onto clean glass slides and stored until further use. Before incubation, sections were air dried for 5 min at room temperature. The incubation medium contained 10% w/v polyvinyl alcohol (PVA), which was dissolved in 100 mM Tris-maleate buffer (pH 8.0). The following compounds were added shortly before incubation of the cryostat sections: 0–12.5 mM DAB, 0–6.5 mM $MnCl_2$ and 0–100 mM $CoCl_2$ (Hsu & Soban, 1982; Steinbeck *et al.*, 1994). All the compounds except DAB were added in strict order and thoroughly mixed from stock solutions to the PVA-containing medium. Incubations lasted for 30 min at $37^{\circ}C$. After incubation, sections were washed in hot distilled water ($60^{\circ}C$) to stop the reaction immediately and to remove the viscous incubation medium. The sections were then mounted in glycerol jelly and studied immediately with a light microscope.

Incubation medium containing 12.5 mM DAB, 2.5 mM $MnCl_2$ and 40.0 mM $CoCl_2$ in 10% PVA in 100 mM Tris-maleate buffer (pH 8.0) was applied to study the source of final reaction product in cryostat sections of liver and small intestine. To investigate whether DAB polymerization was enzyme mediated, the following experiments were carried out: (1) preheating sections in distilled water for 10 min at $37^{\circ}C$, $65^{\circ}C$ and $80^{\circ}C$; (2) prefixing sections for 5, 15 and 60 min in 4% w/v formaldehyde in 100 mM sodium cacodylate buffer (pH 8.0); and (3) varying incubation time (15–60 min).

Characterization of the enzymes responsible for the formation of final reaction product was performed by adding one of the following substrates to the incubation medium: 0.1–2.5 mM NADH (NADH oxidase), 0.1–2.5 mM NADPH (NADPH oxidase), 0.5 mM hypoxanthine (xanthine oxidase) or 0.4 mM cytochrome *c* (cytochrome *c* oxidase) or by adding the following inhibitors: 10 mM 3-amino-1,2,4-triazole (catalase), 1 or 5 mM KCN (catalase, peroxidase, cytochrome *c* oxidase, superoxide dismutase), 5.0 mM NEM (blocker of SH groups), 10 mM PCMB (blocker of SH groups), 0.5 mM $(NH_4)_2S$ (cytochrome *c* oxidase), 1 mM allopurinol (xanthine oxidase), 2.5 mM NAD^+ (NADH oxidase), 2.5 mM $NADP^+$ (NADPH oxidase), 0.01 mM rotenone or 0.1% w/v Triton X-100 (NADH: coenzyme Q reductase) and 5 mM menadione or 0.5 mM disulphiram (aldehyde oxidase) (Zollner, 1989). The involvement of oxygen in the reaction was studied by incubating sections in media saturated with nitrogen, pure oxygen and air using a tonometer (Best *et al.*, 1990).

The type of ROS that was generated in sections after incubation was established by using the following more or less specific ROS quenchers or scavengers: 5–5000 units catalase or 100–1000 units SOD (both spread as a film of enzyme dissolved in 10 μ l of distilled water on glass slides on top of which sections were adhered); 5.0 mM

NaN₃, 5.0 mM histidine, 5.0 mM benzoquinone or 5.0 mM mannitol (Table 3).

Electron microscopy

Cryostat sections were incubated with the semi-permeable membrane technique (McMillan, 1967), which prevents leakage of soluble compounds from the sections during incubation and preserves the ultrastructure of unfixed cryostat sections during incubation (Schellens *et al.*, 1992; Van den Munckhof *et al.*, 1994; Song *et al.*, 1995). The incubation medium contained 12.5 mM DAB, 2.5 mM MnCl₂, 0 or 40.0 mM CoCl₂ and 1% w/v Agar Noble in 100 mM Tris-maleate buffer, pH 8.0. Preparation of the semi-permeable membranes and the incubation vessels was performed as described in detail before (Schellens *et al.*, 1992; Van den Munckhof *et al.*, 1994). After pouring the media into the incubation vessels, they were allowed to solidify at room temperature. Subsequently, cryostat sections were mounted on the semi-permeable membrane. In this way, the semi-permeable membrane separates the section from the gelled medium. Incubations lasted for 30 min at 37°C. After incubations, the gelled media were removed with a spatula. Parts of the semi-permeable membrane with adhering cryostat sections were cut out and fixed immediately for 60 min at room temperature in a mixture of 1% w/v glutaraldehyde and 4% w/v formaldehyde dissolved in 100 mM sodium cacodylate buffer, pH 7.4. Then, sections were rinsed in buffer, post-fixed for 60 min in 1% OsO₄ in 100 mM sodium cacodylate buffer, pH 7.4, and rinsed again. Dehydration and embedding in epoxy resin LX 112 were accomplished following routine procedures. After polymerization, semi-thin and ultrathin sections were cut. Ultrathin sections were placed on grids carrying a Formvar film and studied either unstained or stained with Uranyl Acetate and Lead Citrate with a Zeiss EM 10c transmission electron microscope.

Results

Final reaction product was generated as a blue DAB-cobalt polymer in rat liver and small intestine after incubation of unfixed cryostat sections in a medium containing DAB, MnCl₂ and CoCl₂ in 10% PVA in Tris-maleate buffer (pH 8.0). Highest amounts of final reaction product were found when the incubation medium contained 12.5 mM DAB, 2.5 mM MnCl₂ and 40 mM CoCl₂, and this composition of the medium was used for all further experiments. Higher concentrations of MnCl₂ and/or CoCl₂ did not increase the staining intensity further. The concentration of manganese ions is five times higher than originally described by Briggs *et al.* (1983), but is not as high as proposed by Babbs *et al.* (1991). Babbs *et al.* (1991) used 40 mM MnCl₂ to compete with native superoxide dismutase when applied to detect oxygen radical formation extracellularly in an isolated perfused heart system.

In liver, final reaction product was found in

hepatocytes in periportal zones, whereas hardly any precipitate was observed in pericentral hepatocytes and in non-parenchymal cells (Fig. 1). Final reaction product was heterogeneously distributed in hepatocytes within one periportal area (Fig. 1). In small intestine, the highest concentration of the DAB-cobalt complex was found at the basal side of enterocytes (Fig. 2). Goblet cells remained unstained. The staining was most intense at the villi and was very weak at the bottom of the crypts. Connective tissue remained unstained, except for intensely stained cells in the lamina propria, which are polymorphonuclear leucocytes. In external muscle layers of the small intestine, a weak staining was visible.

The effects of the composition of the incubation medium on the formation of final reaction product in liver and small intestine were as follows. Addition of DAB alone did not result in formation of final reaction product, neither did Mn²⁺ or Co²⁺. Mn²⁺ added to DAB did not induce any final reaction product formation. However, Co²⁺ and DAB did induce the formation of blue final reaction product (Fig. 1A) and the amount was greatly increased by the addition of manganese ions (Fig. 1B). Under these conditions, similar localization patterns of precipitate were observed in both liver and small intestine.

Formation of final reaction product was not affected by preincubation of the sections in an aqueous medium at 37°C, but preincubation at 65°C and 80°C resulted in a complete inhibition of final reaction product formation. Prefixation of the sections also abolished formation of final reaction product completely. The amount of final reaction product increased with increasing incubation times from 15 to 60 min. It was concluded from these results that it is likely that tightly bound enzyme activity was responsible for the formation of precipitate.

The involvement of oxygen as substrate in the reaction was established by incubating sections in media saturated with 100% nitrogen, 100% oxygen or air (20% oxygen). Highest amounts of final reaction product were found when sections were incubated in the presence of 100% oxygen, and no reaction product was formed after incubation in 100% nitrogen. All further experiments were carried out in air for reasons of easiness.

Table 1 shows the effects of various oxidase substrates on the reaction. Addition of low concentrations of NADH or NADPH, substrates of NADH and NADPH oxidase, respectively, did not affect the reaction, but high concentrations resulted in a significant inhibition of the formation of DAB-cobalt complex. Addition of hypoxanthine, a substrate of xanthine oxidase, did not show any effect.

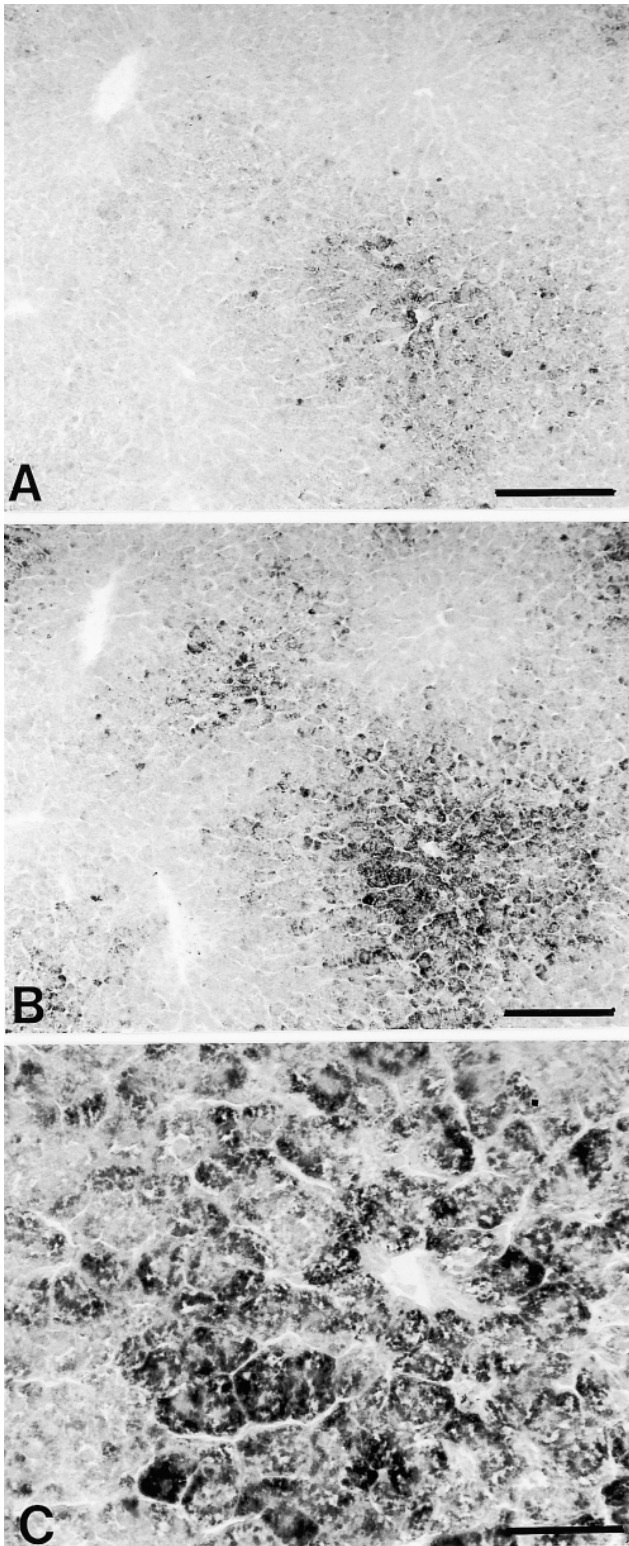


Fig. 1. Photomicrographs of serial cryostat sections of rat liver incubated with a DAB- Co^{2+} - (A) or a DAB- Mn^{2+} - Co^{2+} -containing medium (B,C). Final reaction product is found in hepatocytes surrounding portal tracts, but not in pericentral hepatocytes and non-parenchymal cells. Addition of manganese ions largely increased the amount of final reaction product. (A and B) Magnification 375. Bar = 200 μm . (C) Magnification 3300. Bar = 50 μm .

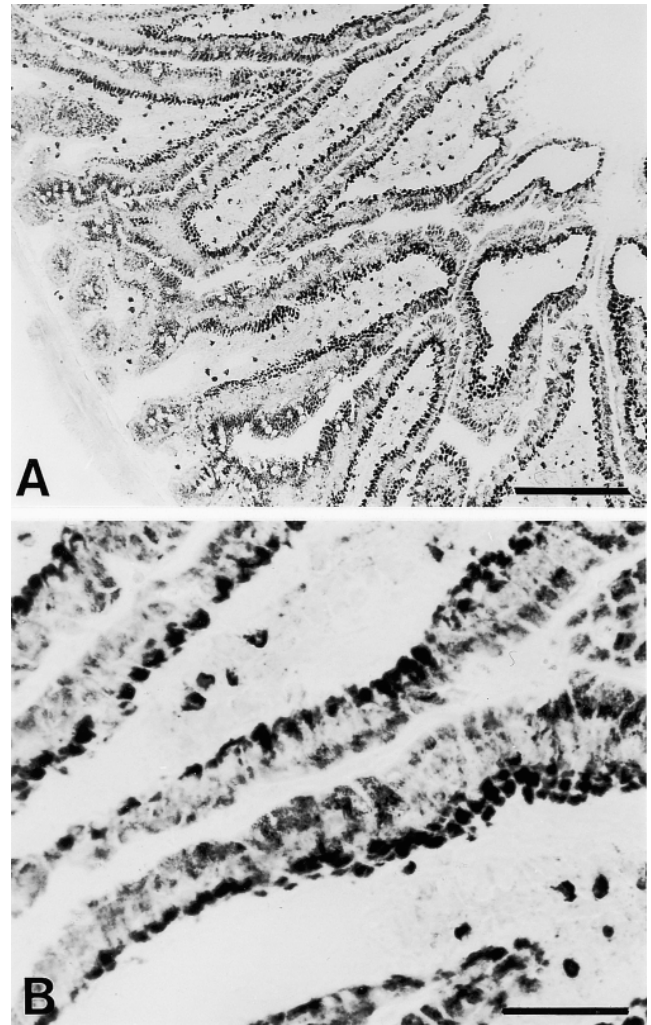


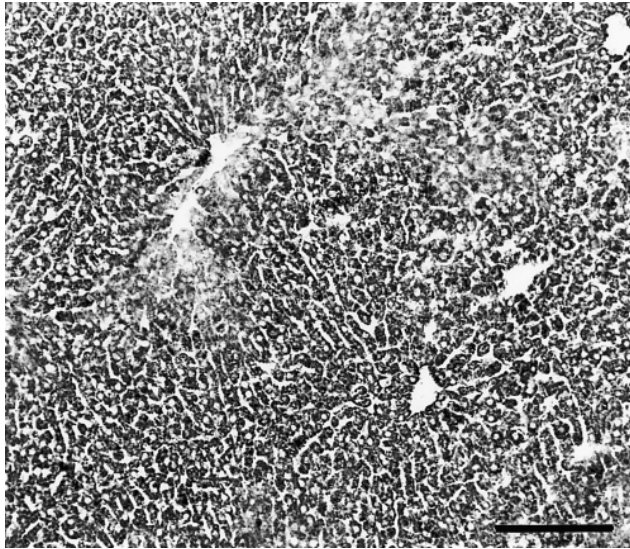
Fig. 2. Photomicrographs of cryostat sections of rat small intestine incubated with a DAB- Mn^{2+} - Co^{2+} -containing medium. Final reaction product is mainly found at the basal side of enterocytes. Staining is most intense at the villi and almost absent at the bottom of crypts. In the lamina propria, positive polymorphonuclear leucocytes are present. (A) Magnification 375. Bar = 200 μm . (B) Magnification 3300. Bar = 50 μm .

Addition of cytochrome *c* to the DAB-Mn-Co medium increased the amount of final reaction product and gave rise to the following localization. In liver, final reaction product was found equally in periportal and pericentral liver parenchymal cells (Fig. 3). In small intestine, final reaction product was formed in cells of the external muscle layers, in epithelial cells of the crypts and at the basal and luminal side of epithelial cells of villi.

The effects of several enzyme inhibitors are shown in Table 2. NEM and PCMB, which inhibit sulphhydryl-containing enzymes, did not affect the reaction. Aminotriazole or allopurinol, inhibitors of catalase and xanthine oxidase respectively, did not reduce the amount of final reaction product.

Table 1. Effects of oxidase substrates in DAB-Mn²⁺-Co²⁺-containing incubation media on the formation of final reaction product in cryostat sections of rat liver and small intestine

Addition	Concentration	Substrate of	Liver	Small intestine
-	-	-	++++	++++
NADH	0.1 mM	NADH oxidase	++++	++++
	2.5 mM		++	++
NADPH	0.1 mM	NADPH oxidase	++++	++++
	2.5 mM		++	++
Hypoxanthine	0.5 mM	Xanthine oxidase	++++	++++
Cytochrome <i>c</i>	0.4 mM	Cytochrome <i>c</i> oxidase	++++++	++++++

**Fig. 3.** Photomicrograph of cryostat section of rat liver incubated with a DAB-Mn²⁺-Co²⁺-containing medium in the presence of 0.4 mM cytochrome *c*. Final reaction product is found equally in periportal and pericentral liver parenchymal cells. (A) Magnification 375. Bar = 200 μ m.

Addition of 1 and particularly 5 mM KCN inhibited the reaction significantly. Ammonium sulphide, a more specific inhibitor for cytochrome *c* oxidase activity, affected the reaction similarly. Addition of NADP⁺, product of NADPH oxidase activity, had no effect, but NAD⁺, product of NADH oxidase activity, reduced the amount of final reaction product by more than 50%. Rotenone and Triton X-100, specific inhibitors of NADH:coenzyme Q reductase, reduced the amount of final reaction product equally. This was also found for menadione and disulphiram, inhibitors of aldehyde oxidase.

The results of incubations of sections in the presence of ROS quenchers and scavengers are shown in Table 3. The presence of exogenous catalase, which uses H₂O₂ as substrate, had no effect in epithelial cells or hepatocytes, but inhibited final reaction product formation in PMNs in the lamina propria. Mannitol scavenges OH \cdot , but did not exert any effect. Also SOD, which uses O₂ \cdot^- as a substrate with concomitant formation of H₂O₂, did not affect the staining intensity. Benzoquinone, a specific scavenger of ROS, prevented the formation of precipitate almost completely. Addition of histi-

Table 2. Effects of enzyme inhibitors in DAB-Mn²⁺-Co²⁺-containing incubation media on the formation of final reaction product in cryostat sections of rat liver and small intestine

Addition	Concentration	Inhibitor of	Liver	Small intestine
-	-	-	++++	++++
NEM	5.0 mM	Sulphydryl groups	++++	++++
PCMB	10.0 mM	Sulphydryl groups	++++	++++
Aminotriazole	10.0 mM	Catalase/oxidase	++++	++++
Allopurinol	1.0 mM	Xanthine oxidase	++++	++++
KCN	1-5 mM	Cytochrome <i>c</i> oxidase	++	++
(NH ₄) ₂ S	0.5 mM	Cytochrome <i>c</i> oxidase	++	++
NADP ⁺	2.5 mM	NADPH oxidase	++++	++++
NAD ⁺	2.5 mM	NADH oxidase	++	++
Rotenone	0.01 mM	NADH-coenzyme Q reductase	++	++
Triton X-100	0.1% (w/v)	NADH-coenzyme Q reductase	++	++
Menadione	5 mM	Aldehyde oxidase	++	++
Disulphiram	0.5 mM	Aldehyde oxidase	++	++

Table 3. Effects of ROS scavengers/quenchers in DAB-Mn²⁺-Co²⁺-containing incubation media on the formation of final reaction product in cryostat sections of rat liver and small intestine

Addition	Concentration	Scavenger of	Liver	Small intestine
-	-	-	++++	++++
Catalase	5-1000 U	H ₂ O ₂	++++	++++
Mannitol	5.0 mM	OH·	++++	++++
SOD	100-1000 U	O ₂ ^{·-}	++++	++++
Benzoquinone	5.0 mM	O ₂ ^{·-}	+	+
Histidine	5.0 mM	O ₂ (¹ ΔG)	++	++
NaN ₃	5.0 mM	O ₂ (¹ ΔG)	-	-

dine, a scavenger of O₂(¹ΔG), led to a dramatic decrease in the formation of final reaction product. Addition of NaN₃ to the DAB-Mn-Co medium abolished the formation of precipitate. Azide is an inhibitor of many enzymes, such as those of the respiratory chain in mitochondria, but may act also as a scavenger of singlet oxygen (Gupta & Rohatgi-Mukherjee, 1978).

Electron microscopy revealed that electron-dense final reaction product was exclusively present in mitochondria in liver parenchymal cells and not in any other cellular structures or in the cytoplasmic matrix (Fig. 4A). Final reaction product was present at both inner and outer membranes and at the intermembrane space between inner and outer membranes of mitochondria (Fig. 4B). Similar observations were made in mitochondria in enterocytes of small intestine (Fig. 4C). In both tissues, some mitochondria contain large amounts of electron-dense reaction product, whereas others do not react at all (Fig. 4A & C). The localization pattern was similar when sections were incubated with a DAB-Mn-containing medium and a DAB-Mn-Co-containing medium.

Discussion

First, experiments were carried out to enhance the sensitivity of the procedure to localize ROS production as described by Graham and Karnovsky (1966) and Briggs *et al.* (1983, 1986) in PMNs. Our results with the use of unfixed cryostat sections of liver and small intestine showed that the sensitivity of the reaction was greatly improved by addition of Co²⁺ to the incubation medium containing Mn²⁺ and DAB. Addition of Co²⁺ to the incubation medium did not affect the localization of final reaction product at the light microscopical or at the electron microscopical level. Therefore, we concluded that cobalt ions enhance the Mn²⁺-DAB reaction as was described previously by Hsu and Soban (1982) for the DAB polymer formed in immunohistochemical procedures. On the other hand, Steinbeck *et al.*

(1993a) reported that Co²⁺ interfered in the oxidation of DAB by Mn³⁺ ions. Experiments were performed to assess whether and which ROS were produced. Addition of catalase did not induce any effect in hepatocytes and intestinal epithelial cells, indicating that H₂O₂ was not the ROS responsible for product formation. Catalase activity was effective, because formation of reaction product by myeloperoxidase in intestinal PMNs did not occur. Hydroxyl radicals were also not produced, because addition of mannitol did not affect the amount of final reaction product.

Results obtained with benzoquinone, a well-known scavenger of several ROS but not of O₂(¹ΔG) (Peters & Rodgers, 1981), showed that ROS were indeed produced. However, exogenous SOD, which uses O₂^{·-} as substrate, did not affect the formation of final reaction product. It cannot be assessed whether the SOD activity was high enough to scavenge O₂^{·-} effectively and whether SOD penetrated mitochondria (Kontos *et al.*, 1992). It is well known that large protein molecules hardly diffuse into unfixed cryostat sections when used as auxiliary enzyme in enzyme histochemical procedures, such as creatine kinase (Frederiks *et al.*, 1988). Therefore, a possible involvement of O₂^{·-} cannot be excluded.

Addition of histidine resulted in a large decrease in the amount of final reaction product but did not affect the staining of PMNs. Histidine is known for its capacity of scavenging O₂(¹ΔG) (Tomita *et al.*, 1969) and has been used in several studies to demonstrate the presence of O₂(¹ΔG) (Hodgson & Fridovich, 1974; Steinbeck *et al.*, 1992, 1993b; Karnovsky, 1994). However, controversy still remains with respect to the specificity of histidine for O₂(¹ΔG). For instance, histidine can react with HOCl, OH· and, very recently, Cai *et al.* (1995) reported that histidine reacted with H₂O₂. Based on the results described above, OH· and H₂O₂ are not likely candidates, whereas HOCl would only play a role in PMNs and not in parenchymal liver cells or in epithelial cells of small intestine. The reactivity of

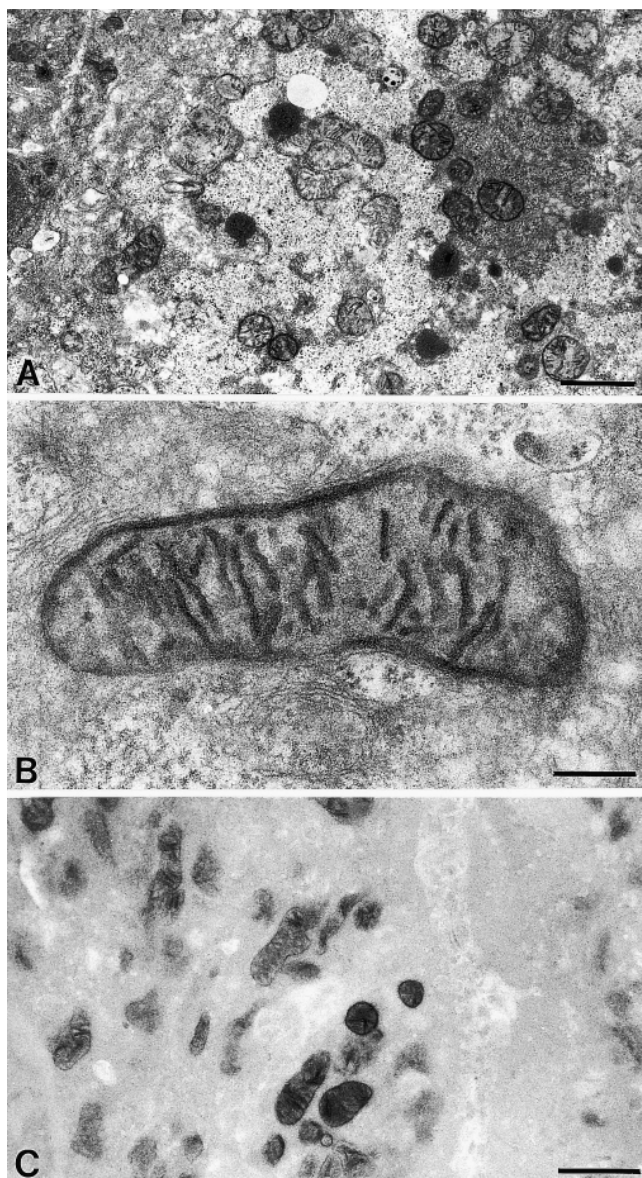


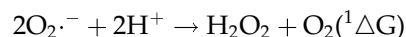
Fig. 4. Electron micrographs of cryostat sections of rat liver incubated with a DAB-Mn²⁺-Co²⁺-containing medium (A and B) and of rat small intestine incubated with a DAB-Mn²⁺-containing medium (C) and the semi-permeable membrane technique. Ultrathin sections were stained with Uranyl Acetate and Lead Citrate (A and B) or were not stained (C). The amount of electron-dense reaction product in different mitochondria varies. Reaction product is localized at inner and outer membranes of mitochondria and at the intermembrane space. (A) Magnification 34410. Bar = 2.5 μ m. (B) Magnification 314000. Bar = 0.75 μ m. (C) Magnification 34410. Bar = 2.5 μ m.

histidine for O₂^{·-} has proved to be minimal (Hodgson & Fridovich, 1974).

The conclusion that O₂(¹ΔG) may be produced is supported by the following data. Sodium azide abolished the reaction completely, which may be

caused by inhibition of cytochrome *c* oxidase but also by the scavenging capacity of sodium azide of singlet oxygen (Gupta & Rohalgi-Mukherjee, 1978). Addition of high concentrations of NADPH and NADH resulted in a severe decrease in the amount of final reaction product in both liver and small intestine. It was reported previously that NADH and NADPH directly scavenge O₂(¹ΔG) (Patriarca *et al.*, 1975; Curnutte *et al.*, 1976), but they also scavenge O₂^{·-} ions via a free-radical reaction chain in the presence of Mn²⁺ ions leading to Mn³⁺ and then formation of a DAB polymer. However, we observed a decrease in the amount of reaction product and, therefore, it is likely that O₂(¹ΔG) is formed in our DAB-Mn-Co reaction.

Two pathways for the production of O₂(¹ΔG) in living cells are known. The first is a myeloperoxidase-dependent mechanism in which formation of HOCl is followed by O₂(¹ΔG) production (Steinbeck *et al.*, 1992; Khan & Kasha, 1963, 1970). However, this mechanism can be ruled out in our system because myeloperoxidase is not active in either parenchymal liver cells or epithelial cells of small intestine. The second is the spontaneous dismutation reaction that can be inhibited by SOD (Corey *et al.*, 1987; Khan, 1970):



The occurrence of this reaction in our system is supported by our findings that benzoquinone, which scavenges O₂^{·-}, but also other free radicals, reduced the reaction considerably. A prerequisite for this reaction in mitochondria is the absence of SOD at the sites at which O₂^{·-} is formed. This may be true because Mn-SOD is localized in the matrix of mitochondria (Slot *et al.*, 1986), whereas final reaction product resulting from the DAB-Mn-Co reaction was present at inner and outer membranes of mitochondria and in the intermembrane space. Although originally the Mn²⁺-DAB cytochemical method was designed to localize specifically sites of O₂^{·-} production, it was found later that the formation of O₂(¹ΔG) can also lead to DAB polymerization (Steinbeck *et al.*, 1993a; Karnovsky, 1994).

We conclude that the generation of DAB-cobalt complex was likely to be linked with tightly bound enzyme activity because preheating or fixation of the sections abolished the reaction completely, pretreatment with aqueous media did not show any effect and formation of final reaction product was time dependent.

Several possible enzymatic sources of ROS were ruled out on the basis of results presented in Tables 2 and 3. First, xanthine oxidase activity is not likely to be the source of ROS in both liver and small intestine because neither allopurinol nor hypox-

anthine resulted in a detectable effect. Moreover, the granular form of final reaction product was not indicative of a soluble enzyme, such as xanthine oxidase (Kooij, 1994). Secondly, the presence of NADP⁺ did not have any effect. Therefore, NADPH oxidase activity could also be ruled out as contributing to DAB-cobalt complex formation. This can also be said for catalase activity, because aminotriazole did not affect the reaction.

It is likely that cytochrome *c* oxidase activity is responsible for part of the generation of final reaction product because KCN and (NH₄)₂S, a specific inhibitor of cytochrome *c* oxidase, reduced the reaction. The localization of final reaction product that was induced by addition of cytochrome *c* was similar to the localization described in the literature (Hiraoka *et al.*, 1986) but differed from the localization after DAB-Mn-Co incubation in the absence of cytochrome *c*. The amount of final reaction product of cytochrome *c* oxidase activity was higher and was found in more cells than after incubation with the DAB-Mn-Co medium. Endogenous cytochrome *c* may be held responsible for the spontaneous formation of final reaction product. The staining intensity caused by DAB and Co²⁺ in the absence of Mn²⁺ may have induced this reaction. However, cytochrome *c* oxidase is not the only candidate responsible for the formation of final reaction product, because in that case DAB polymerization occurs without involvement of ROS, whereas it has clearly been demonstrated that O₂⁻ and O₂(¹ΔG) play a role.

Other experiments performed to determine the type of enzyme responsible for the formation of ROS showed that (1) sulphhydryl groups were not essential; (2) the enzyme was NAD⁺ sensitive; (3) the enzyme was inhibited by rotenone and Triton X-100; and (4) the enzyme was inhibited by menadione and disulphiram. These results indicate that NADH:coenzyme Q reductase and aldehyde oxidase are possible candidates (Kehrer, 1993). The ultrastructural localization of final reaction product at inner and outer membranes of mitochondria and at the intermembrane space also support the conclusion that the proposed enzymes are involved (Hiraoka *et al.*, 1986; Critchley *et al.*, 1992).

Summarizing, we believe that the DAB-Mn-Co-containing incubation medium gives rise to the formation of final reaction product at inner and outer membranes of mitochondria in liver parenchymal cells and epithelial cells of small intestine. Reaction product formation is partly caused by cytochrome *c* oxidase using endogenous cytochrome *c* and is partly due to superoxide anion production by NADH:coenzyme Q reductase and aldehyde oxidase followed by spontaneous singlet oxygen formation.

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