ULTRASTRUCTURAL DEMONSTRATION OF NADPH OXIDASE
ACTIVITY IN KUPFFER AND ENDOTHELIAL CELLS
USING AN ISOLATED LIVER PERFUSION SYSTEM
—COMPARISON BETWEEN ICR/D-STRAIN AND
JCL: WISTAR-STRAIN RATS—

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In order to cytochemically demonstrate the NADPH dependent H_2O_2 generation system in the sinusoidal lining cells of the liver, the authors applied the modified method of Briggs *et al.* (5) to an isolated liver perfusion system devised by Sugano *et al.* (21). The rat liver was perfused with the reaction medium containing 40 mg NADPH, 40 mg $CeCl_3 \cdot 7H_2O$ and 2 mg glucose in 100 ml of 0.1 M tris-maleate buffer solution (pH 7.4), continuously supplied with a gas mixture (95% O_2 and 5% CO_2). With respect to the specificity of the reaction, the *in situ* localization, and the amount of reaction products, adequate results were obtained with this system. Thus, the activity in the sinusoidal lining cells was compared between livers of the Jcl:Wistar- and the ICR/d-strain rats affected with spontaneously occurring cataracts and dermatitis.

Both Kupffer and endothelial cells showed conspicuous reactivity. A specific and significant reaction product was observed, adherent to the plasma membrane, on the external cell surface, including that of invaginated portions or coated pits. Furthermore, it was also noted on the internal surface of phagocytic vacuoles or coated vesicles. Comparing the two strains of rat, a much higher reactivity was shown in the liver of ICR/d-rat, suggesting that a defensive activity may be enhanced in the sinusoidal area to compensate for constitutional defects such as immunological disorders inherent in the ICR/d-strain.

A free radical generation system (hydrogen peroxide and superoxide etc.) in leukocytes and macrophages plays an important role in bactericidal or detoxifying activities through phagocytosis (1, 3, 6, 15, 17). For the activity of NAD(P)H-dependent, cyanide insensitive H_2O_2 generation (NAD(P)H oxidase) (1, 16, 18, 22), a cytochemical technique for localization of H_2O_2 generated was developed by Briggs *et al.* (5). Recently, this method was also applied to the demonstration of plasma-membrane associated NAD(P)H oxidase activity in pulmonary macrophages by Hirai *et al.* (10).

Abbreviations in this paper: NADPH, beta-Nicotinamide adenine dinucleotide phosphate, reduced form; PM, Standard perfusion medium; RM, Standard reaction medium; LPS, Lipopolysaccharide.

This oxidative activity might also be functioning in the resident macrophages, such as the Kupffer cells, which play an important role in the sinusoidal lumen of the liver. In order to evaluate this possibility, the authors modified the method of Briggs *et al.* (5), and applied it to an isolated liver perfusion system devised by Sugano *et al.* (21) for ultrastructural detection of the specific reaction. Reaction products were found on the plasma membrane of the Kupffer cells as well as on the endothelial cells under conditions in which the histological and cytological architecture was well maintained.

This report is concerned with the specificity of reaction in this system, reactive cell types and sites at the ultrastructural level, with particular emphasis on the pathological findings seen in the ICR/d-strain rat.

MATERIALS AND METHODS

Experimental animals

(1) Male rats of ICR/d-strain aged 2 to 3 months were used. This strain of rat (11–13), which has spontaneously occurring cataract and dermatitis were bred and maintained in our laboratory under conventional conditions, having free access to food and water.

(2) Rats of Jcl : Wistar strain of the same sex and age were used after being reared under the same conditions for at least one week after purchase.

Each experimental group consisted of 10 to 16 animals. However, for examination of the specificity of the reaction, mainly the ICR/d-strain rats were used, because of the higher reactivity in their sinusoidal lining cells.

Methods

1. Pre-perfusion stage

For the present experiment, the liver perfusion system (Sugano *et al.*) (21) was applied, with a hemoglobin-free medium (Fig. 1). Procedures of liver extirpation

FIG. 1. Scheme of outline of liver perfusion system used in this experiment.

and its setting to the perfusion apparatus have been described in detail by Sugano *et al.* (21) and by Funo (8).

The composition of the standard perfusion medium (PM) is shown in Table 1, as follows.

TABLE 1. *Composition of the standard perfusion medium (PM)*

KCl	5.9 mM
MgCl ₂	1.2 mM
NaH ₂ PO ₄	1.2 mM
Na ₂ SO ₄	1.2 mM
CaCl ₂	2.5 mM
NaCl	115.0 mM
NaHCO ₃	25.0 mM
Glucose	10.0 mM

The PM was kept at 32°C and supplied continuously with a fine foamy gas mixture (95% O₂ and 5% CO₂). In each experiment, pre-perfusion with the standard PM was performed for 30–40 min, because it has been found that the extirpated liver is restored to its physiological state after such a procedure (21).

2. Detection stage

(1) Perfusion procedure with the standard reaction medium (RM).

These procedures are described in sequential order as follows:

All solutions were prepared just before use, and supplied continuously with the gas mixture.

a) After pre-perfusion, the sinusoidal lining cells were stimulated through a closed circulation system with the standard PM containing lipopolysaccharide B of *Escherichia coli* (LPS, 055:B6, Difco Lab. Detroit Mich.) at a concentration of 10 mg/100 ml for 10 min.

b) After a rinsing perfusion with a physiological salt solution, the liver was perfused with the standard RM shown in Table 2, as follows.

TABLE 2. *Composition of standard reaction medium (RM)*

NADPH	40 mg
CeCl ₃ ·7H ₂ O	40 mg
Glucose	2 mg
in	
0.1 M tris-maleate buffer	
100 ml (pH 7.4)	

Note: 3-amino-1,2,4, triazole, an anti-catalase agent was not used in this RM, because, on exposure to the agent, the liver swelled remarkably and thereby blocked further perfusion.

Perfusion time was about 3 min and 20 sec (100 ml of the RM was used as a whole) in the case of ICR-rats and about 6 min and 40 sec (200 ml as a whole) in the case of Jcl:Wistar rats respectively.

c) Rinsing perfusion with a physiological salt solution.

d) Fixing perfusion with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 to 3 min.

Tissue blocks of about 1 × 1 mm³ were obtained from the right central lobe

immediately after the final perfusion, immersed again in the same fixative for an additional 30 min, post-fixed with 1% OsO₄ in 0.1 M veronal buffer (pH 7.4) after rinsing with 0.1 M cacodylate buffer solution and processed routinely for electron microscopic observation. For preparation of ultrathin sections, periportal areas of the hepatic lobule were selected.

(2) Reactivity tests under a few abnormal conditions

The following two tests were performed in order to observe (a) a strict oxygen dependency of this reaction system and (b) a substantial stimulation effect of LPS.

a) Cutting off the supply of the gas mixture during the period when the liver was perfused with the RM.

b) Omission of the stimulation procedure with LPS.

(3) Specificity tests

A series of control experiments was performed in order to gain some insight into the specificity of the reaction.

a) Perfusion with the reaction medium (prepared at pH 7.4) from which the substrate (NADPH) or CeCl₃ was eliminated.

b) Pre-fixation before exposure to the RM through perfusion with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 sec, 1 or 2 min.

c) Addition of catalase (manufactured by Sigma Chem.) to the standard RM (pH 7.4) at concentrations of 10, 20 and 30 mg/100 ml.

d) Addition of KCN to the standard RM (pH 7.4–7.6) at concentrations of 10, 20 and 40 mg/100 ml.

e) Perfusion with a buffered medium containing fine cerium precipitate (passed through 45 micron milipore filter), previously prepared by artificial addition of H₂O₂ to the CeCl₃ solution. The detailed procedures have been described by Funo (8).

RESULTS

1. Activity findings in the ICR/d-strain

(1) H₂O₂ generation and localization in the standard procedures

Both the Kupffer and the endothelial cells showed conspicuous reactivity.

In the Kupffer cells, the external surface of the plasma membrane including the invaginated regions or coated pits, and more notably the side bordering on the sinusoidal lumen, was covered widely with a layer of cerium precipitate (Fig. 2). In some regions, this layer was continuous and in other regions it was discontinuous. Additionally, phagocytic vacuoles as well as the apparent profiles of surface-connected channels were lined with the reaction products in a fashion similar to that seen on the outer cell surface. However, vacuoles containing a large or small amount of LPS showed little or only scattered reaction products on their internal surface (Figs. 2, 3). Free reaction products, which did not adhere to the plasma membrane, were also observed intermittently in the sinusoidal lumen, especially in the vicinity of the cell border, and also in the pit-like or invaginated portions, and phagocytic vacuoles (Fig. 3). Golgi apparatus, endoplasmic reticulum, mitochondria and other subcellular structures showed no reaction products.

In the endothelial cells, the external surface of the cell membrane, pit-like portions and coated pits, and the internal surface of coated or pinocytic vesicles

showed a conspicuous layer of the precipitous deposits similar to that seen in the Kupffer cells (Fig. 4a). Especially on the external surface of coated pits, a notable reaction could be seen, frequently displaying a localized thick layer of cerium precipitate (Fig. 4b). Another notable finding was that, when the endothelial cells or endothelial cells and a Kupffer cell were closely connected with each other, a conspicuous and continuous deposit of the reaction products was observed on the extensive interface of the cell membranes (refer to Fig. 5).

(2) Reactivity under a few abnormal conditions

a) When the supply of the gas mixture was stopped during perfusion of the standard RM, the reactivity was strongly suppressed in both cells of the sinusoidal lining so that almost no precipitate could be seen (Fig. 6).

b) When the stimulation procedure with LPS was omitted, the reaction products showed a significant decrease at the reactive sites of both cell types, although a weak or moderate precipitation was observable (Fig. 7).

(3) Examination for specificity of the reaction

a) When CeCl_3 was eliminated from the standard RM, almost no reaction could be seen.

When the substrate (NADPH) was eliminated from the standard RM, the amount of cerium precipitate was dramatically reduced, showing only slight deposition in the normally reactive sites (Fig. 8).

It is therefore clear that the generation of reaction products is strongly dependent on the presence of exogenous NADPH.

b) Exposure to glutaraldehyde before perfusion with the RM resulted in total elimination of reaction products from the cell (Fig. 9). At the same time, almost no precipitate was observed, while otherwise it would have been seen free in the sinusoidal lumen, cellular cavities or phagocytic vacuoles.

c) When catalase was included in the standard RM at concentrations of 20 or 30 mg/dl, cerium precipitation was almost completely suppressed (Fig. 10). At a concentration of 10 mg/dl, only some areas showed a reduced amount of reaction products.

d) When KCN was included in the RM at concentrations of 10 or 20 mg/dl, no notable inhibition occurred. Even when the concentration was increased to 40 mg/dl, shifting the pH of the medium slightly to the alkaline side (pH 7.6), there was almost no interference with the generation of precipitate (Fig. 11).

e) The possibility of a preferential binding of reaction products to the Kupffer or the endothelial cells was investigated by perfusing with the cerium medium already precipitated by prior addition of H_2O_2 , instead of normal perfusion with the RM. The result was that reaction products did not adhere closely and evenly to the cell membrane of the normally reactive sites, an appearance quite distinct from that with the proper perfusion (Fig. 12).

2. The activity findings in the Jcl: Wistar-rat compared with those in the ICR/d-rat

Under standard experimental conditions, it was found that both cell types of the hepatic sinusoid in Jcl: Wistar rats showed a comparatively weak reaction. Therefore, the liver of this strain was perfused with an additional 100 ml, plus 100 ml of the RM and the duration was doubled. As a result, generation of precipitate was significantly increased to a level largely similar to that seen in the livers of ICR/d-strain.

Abbreviations used in the following figures: S, sinusoid; N, nucleus, V, phagocytic vacuoles; P, coated pits; K, Kupffer cell; E, endothelial cell; L, liver cell.

FIG. 2. Kupffer cell of the ICR-rat liver, perfused with perfusion medium containing LPS and then with standard reaction medium (Standard procedures). Double-stained with uranyl acetate and lead citrate. A layer of reaction product (arrowheads) is shown extensively on the external cell surface including invaginated portions. In phagocytic vacuoles containing a moderate or small amount of LPS (small arrows), no clear reaction is noted. However, in the coated-pits or invaginated portions, coexistence of the precipitate with LPS can be seen (large arrow).
× 27,000

FIG. 3. Kupffer cell of the ICR-rat liver, perfused according to the standard procedures. Free reaction product (F mark) is seen in the sinusoidal lumen and also in the invaginated portions or phagocytic vacuoles. At the same time, a layer of precipitate is seen on the outer cell surface or inner surface of the vacuoles (arrowheads), adhering to the plasma membrane. Note the presence of membrane-like LPS (small arrows) in the sinusoidal lumen as well as in the phagocytic vacuoles.
× 27,200

FIG. 4a. Endothelial cells of the ICR-rat liver, perfused according to the standard procedures. Extensive deposits of reaction product are seen on the external cell surface (arrowheads). A relatively thick layer of the precipitate is observed on the outer surface of the coated pits.
× 19,100

FIG. 4b. This photograph shows a high power view of an endothelial cell of the ICR-rat liver, perfused according to the standard procedures. There are coated pits and vesicles (arrowheads) which exhibit an especially thick layer of the precipitate on their external or internal surface.
× 59,200

FIG. 5. This specimen shows endothelial cells and a Kupffer cell of the Jcl:Wistar rat liver, perfused according to the standard procedures. The Kupffer cell, located nearly at the center of the photograph, contains many organelles (LPS-containing phagosomes are not seen in this section). On the extensive interface between the membranes of those which are in close contact with each other, a conspicuous and continuous deposit of reaction products can be seen (arrows). This is one of the characteristic findings obtained in this histochemical experiment.
× 18,600

FIG. 6. Kupffer cells of the ICR-rat liver, perfused according to the standard procedures, but with the gas mixture being shut off during the period of perfusion with the reaction medium. No fixed or free deposits are observed.
× 9,500

FIG. 7. Kupffer cells of the ICR-rat liver, perfused with the standard RM, but LPS stimulation procedure was omitted. Only slight deposits occur on the external surface as shown by the arrowheads.
× 5,800

FIG. 8. Kupffer cell of ICR-rat liver, perfused with RM containing no substrate (NADPH). A relatively small amount of deposit is present only in a few vacuolar or invaginated structures of this cell as shown by the arrows. No reaction products are seen on the external surface.
× 19,500

FIG. 9. Kupffer and endothelial cells of ICR-rat liver, exposed to glutaraldehyde for 30 min before perfusion with the standard RM. No reaction products are observable at any reactive sites of the two cell types.
× 7,800

FIG. 10. Endothelial cell of the ICR-rat liver, perfused with RM containing catalase at a concentration of 20 mg/dl. Reaction products are not seen in the normally reactive sites, for example, in the coated pits shown by the arrowheads.
× 19,000

FIG. 11. Kupffer cells of ICR-rat liver, perfused with the RM containing KCN at a concentration of 40 mg/dl. Even in this condition numerous reaction products are found on the external surface as shown by the arrowheads.
× 18,800

FIG. 12. This specimen shows the Kupffer cell and the sinusoidal lumen of a liver of the ICR-rat, perfused with a buffered medium containing fine cerium precipitates prepared artificially. The precipitates, which are seen in a cluster in this area, never show any tendency to adhere to the cell membrane, although they are partially phagocytized.
× 15,800

FIG. 13. Kupffer cell of the Jcl:Wistar rat liver, perfused according to the standard procedures. The reaction products are seen only in the intracytoplasmic portions of this cell, such as deep cavities or invaginated channel structures and/or phagocytic vacuoles rather than on the external surface as shown by the arrows.
× 13,600

FIG. 14. Endothelial cells of the Jcl:Wistar-rat liver, perfused according to the standard procedures. A thick layer of deposit is seen on the external cell surface, although it is moderately discontinuous. Note a conspicuous reaction on the membrane surface of the coated pits and vesicle (arrow). $\times 39,600$

When the stimulation procedure with LPS was omitted, reaction products were significantly decreased, providing a finding similar to that in the ICR/d-strain under the same conditions. In order to observe the duration of the stimulatory effect of LPS, a 20 min perfusion procedure with the PM was added after the stimulation procedure with LPS; such effect of LPS on the precipitation of reaction products was almost completely abolished (preliminary data).

With regard to details as to the reactivity shown by each cell type, characteristic findings were as follows: Kupffer cells frequently had deposits of cerium precipitate only in the intracytoplasmic areas such as deep cavities, invaginated channel structures and/or phagocytic vacuoles rather than on the external cell surface. This precipitation may have occurred *in situ*, or have already formed on the surface and then quickly internalized or phagocytized (Fig. 13). As for the endothelial cells, no difference between the two strains was observed in the amount and/or the localization of the reaction when the experiment was performed under such conditions as described above (Figs. 5, 14).

DISCUSSION

The present experiment presents strong evidence that the H_2O_2 generation system plays a substantial role in the region of the hepatic sinusoid and additionally, that both cell types, the Kupffer cells and the endothelial cells in this area are equally involved.

With regard to the sensitivity of our detecting system for H_2O_2 generation at the ultrastructural level, the following two points must be considered.

- 1) Under the experimental conditions, it is questionable as to whether or not the sinusoidal lining cells preserve their capacity to generate H_2O_2 dependent on NADPH oxidase activity.
- 2) As a cytochemical technique, deposition of cerium precipitate may not accurately reflect the amount or the *in situ* localization of H_2O_2 generation dependent on NADPH oxidase activity.

Availability of a liver perfusion system employing a hemoglobin and albumin-free medium, was evaluated by Sugano *et al.* (21) from the biochemical, pharmacological and ultrastructural aspects. We also confirmed that the morphological integrity of the sinusoidal lining cells as well as the hepatocytes were well maintained through the perfusion with the PM and the RM. Furthermore, as revealed in this experiment, both cell types showed a marked negative response to the reduced supply of oxygen, although damage at the ultrastructural level due to hypoxia, such as vacuolar degeneration and/or mitochondrial swelling etc. were not observed. This observation is related not only to cellular viability but also to the specificity of the reaction (7, 15–17), for it has been reported that endophagocytosis (as well as appropriate soluble or particulate stimuli to the cell membrane (9, 15)) evokes a marked increase in oxygen consumption due to the enhancement of cyanide-insensitive NAD(P)H oxidase activity (respiratory burst) (15–17, 20). The positive response to LPS stimulation provides further evidence for the reaction specificity.

Next, we will comment on the constituents of the standard RM. In our experimental system, 3-amino-1,2,4-triazole was eliminated from the complete medium proposed by Briggs *et al.* (5) for the reason described previously. However, so far as this perfusion system is concerned, this did not affect the reactivity or its specificity and on the contrary, only 3 min or more was required for adequate generation of precipitate in the case of ICR/d-rat strain.

On the other hand, the Jcl:Wistar rats required a twofold amount of perfusion medium (about 6 min and 40 sec) for evoking a similar reactivity level. This was perhaps due to their comparatively low level of reactivity or sensitivity to LPS stimulation, though a comparatively high H_2O_2 -degrading potential (such as catalase activity etc.) inherent in this strain should not be precluded.

Specificity was further examined by the following test:

Effects of glutaraldehyde

A 30 sec perfusion with 2.5% glutaraldehyde solution completely abolished the cerium precipitation not only on the membrane surface but also in the lumen, be it sinusoidal or intracytoplasmic. This shows a higher sensitivity of the reaction to pre-fixation.

Substrate (NADPH) dependence

When NADPH was eliminated from the RM, the amount of cerium precipitation was markedly reduced in all reactive sites including the sinusoidal lumen, so that only a slight deposition appeared on the membrane surface. It is clear that the formation of reaction product is strongly dependent on the presence of exogenous NADPH (2, 5, 16, 18, 22), whereas occurrence of a slight precipitation suggests the possibility that endogenous NADPH may participate in the reaction.

Cyanide insensitivity

Even if KCN was included in the standard RM at a concentration of 10 mg (1.54 mM), 20 mg (3.07 mM) and 40 mg/dl (6.15 mM), both cell types showed similar amounts and distribution of reaction products to the RM lacking KCN. This is consistent with the failure of KCN to interfere with the respiratory burst associated with phagocytosis or membrane stimulation (15, 17, 20).

Effect of catalase on generation of reaction products

To further confirm if cerium precipitation was dependent on H_2O_2 generation, catalase was added to the standard RM at a concentration of 10 mg, 20 mg or 30 mg/dl. The results showed that cerium precipitation is indeed due to a reaction with H_2O_2 , which can be strongly interrupted by catalase (5).

Perfusion experiment with exogenously generated reaction products

The precipitate produced artificially did not adhere closely and evenly to the plasma membrane, whether it was present on the cell surface or in the vacuoles. This finding shows that distribution of the precipitate is quite different from that occurring in the endogenous H_2O_2 generation system (5).

Regarding the *in situ* localization of reaction products, considerable evidence demonstrates that superoxide anion and successive hydroperoxide production take place on the outer surface of the cell membrane which is then invaginated to form phagocytic vacuoles (1, 4, 9, 22), showing here a bactericidal function or detoxifying action. Our findings are well consistent with this evidence. However, in our experiments, little or only scattered cerous precipitate was observed on the inner surface of the phagocytic vacuoles containing a large or small amount of LPS. A clear explanation for this is impossible now, but the following two possibilities may be considered; that is, $CeCl_3$ is unable to easily penetrate into reactive sites (5), or H_2O_2 is more or less consumed as a result of H_2O_2 or O_2^- reacting with LPS instead of $CeCl_3$.

There is another problem on which we will comment here. This is on the finding that free reaction products without adherence to the plasma membrane occurred frequently in the sinusoidal lumen. The question is whether this was the result of real enzyme activity or not. On detailed observation, it was found that the amount of free products usually paralleled the amount of those closely adherent to the cell membrane under various experimental conditions. Therefore, the following possibilities should be considered: That the precipitate was freed from the surface of the cell membrane after the reaction occurred *in situ*; or that H_2O_2 or O_2^- itself was freed from the cell membrane where it was just generated (2, 6, 15, 19) and the reaction occurred thereafter.

In conclusion, it should be specially emphasized that the endothelial cells, in addition to the Kupffer cells, are found to have a real H_2O_2 generation system dependent on NADPH. This reaction occurred especially on the membrane surface of the coated pits and vesicles, as well as on the extensive interface between a pair of cell membranes of the endothelial cells which were closely connected with each other. All these findings suggest that they may be likewise responsible to a defensive activity.

In comparing the findings between the ICR/d-strain and the Jcl : Wistar-strain (normal controls), it is clear that H_2O_2 generation observed in the sinusoidal lining cells of the former is kept in an enhanced condition, perhaps due to constitutional

defects of the defense mechanisms as represented by frequent occurrence of dermatitis and/or pneumonia (13). It has been also found that this rat strain has a clear tendency to succumb to liver shock when small amounts of LPS are injected intraperitoneally, e.s. 0.3 mg/kg level (11).

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