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TIME KINETICS OF HEMOGLOBIN AND MYOGLOBIN ACTIVATION BY TETRACHLORODECAOXIDE (TCDO)[1]

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In the presence of peroxidase, myoglobin or hemoglobin, Tetrachlorodecaoxide (TCDO) forms an active oxygen species which is similar to the product of the polymorphonuclear leucocyte (PMNL) myeloperoxidase reaction and the 'Klebanoff Model' of phagocytosis, but it is also produced under anaerobic conditions. Randomly destructive species such as the free $OH^{\cdot}$ radical or singlet oxygen are not formed. The kinetics of the heme-dependent activation vary according to the heme type present. In comparison to myoglobin, blood shows a 2 h delay in the appearance of maximal activity. On the basis of known biochemical and clinical-physiological data, a hypothesis can be proposed to explain the reoxygenation observed in hypoxic tissue, induced by TCDO via this activated heme species. Under normal physiological conditions, vasodilation occurs via catalysis by xanthine oxidase or PMNL-dependent activation of fatty acids.

ABBREVIATIONS

OF, Oxoferin; ACC, 1-aminocyclopropane carboxylic acid; POD, peroxidase; KMB, S-methyl-α-ketobutyric acid; Hb, hemoglobin; Myo, myoglobin; TCDO, Tetrachlorodecaoxide.

Key words: Oxoferin[R], Tetrachlorodecaoxide, activated oxygen, wound healing, peripheral oxygen supply.

INTRODUCTION

In clinical studies, it has been demonstrated that the Tetrachlorodecaoxygen complex, TCDO increases the oxygen supply in hypoxic peripheral tissue and also acts as a bactericide[1]. TCDO has been shown in *in vitro* studies under the catalysis of certain

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hemeproteins such as peroxidase(s), myoglobin, Fe^{2+} or Fe^{3+} hemoglobin (but not with catalase) to lead to the formation of an active oxygen species. This agent does not posses the same biological and chemical properties as

a) the aggressive free $OH^{\cdot}$ radical

b) the superoxide anion, $O_2^{\cdot -}$

c) hydrogen peroxide, H_2O_2

d) singlet oxygen, $^1O_2$ (2, 3).

The properties of the TCDO-catalysed heme activation can be determined by the use of indicator reactions such as those with S-methyl-α-ketobutyric acid (KMB) or with 1-aminocyclopropane-1-carboxylic acid, which both lead to the production of ethylene which can be monitored by gas chromatography[2]. In addition to the bactericidal (bacteriostatic) properties[4] TCDO also leads to increased blood supply, which likewise appears to occur via the agency of certain active oxygen species[5]. However, such active oxygen species can also result in undesirable side effects on the tissue and thus, it was of importance to analyse the biochemical and chemical reactivity of the above mentioned TCDO-hemeprotein complex.

All the studies to date tend to indicate a species which appears to have characteristics in common with Compound I of peroxidase or the so-called 'Klebanoff system'[6].

We present here the results of studies on the comparison of the time-dependent activation by hemoglobin, myoglobin, peroxidase and blood. As a result of these investigations, it is possible to propose a hypothesis to account for the increase in oxygen supply in hypoxic tissue and which also explains the bactericidal properties of the drug.

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MATERIALS AND METHODS

The formation of ethylene from the methionine derivative, KMB was determined by gas chromatography[7]. TCDO was incubated with the various hemeproteins in Fernbach vessels with a volume of approx. 15 ml, which were fitted with serum rubber stoppers. After incubation, 1 ml samples of the vessel headspace were taken and analysed for ethylene.

The details of the incubation conditions are given in the legends to the tables and figures. All experiments were repeated three times, each time with parallel incubations. The trends were always the same, although the basic activity varied by up to 30%. Accordingly, the data in the tables and figures show representative experiments.

RESULTS

1) Activated oxygen under anaerobic conditions

As we have recently demonstrated[2], the incubation of TCDO (10 μl of a 158 mM solution) with peroxidase (10 units) for 45 min in the presence of either ACC or KMB results in the formation of 1.5–2 nmol of ethylene. This reaction also occurs under strict anaerobic conditions (N_2-gassed vessels with methyl viologen/dithionite in the

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TABLE I
TCDO-dependent production of ethylene from ACC under anaerobic conditions

Additions	pmol ethylene/45 min
ACC + TCDO	32
ACC + POD	5
ACC + TCDO + POD	1870

Reaction conditions: the reaction mixture contained in 2 ml: 50 mM phophate buffer pH 7.8; 1 mM ACC; 10 U POD and 10 μl TCDO, corresponding to 1.6 μmol ($Cl_4O_{10}^{4-}$), where indicated. The vessels were flushed with N_2, in the centre well were 10 mM sodium dithionite and 1 mM methyl viologen, the blue colour of which was used as an indicator of anaerobic conditions.

TCDO-dependent production of ethylene from ACC under aerobic conditions

Additions	pmol ethylene/45 min
ACC + TCDO	20
ACC + TCDO + POD	1668
ACC + TCDO + 10^{-5}M hemoglobin (Sigma)	1525

Reactions conditions: see above, but carried out in air

centre well), with rates which were the same as in air (Table).

Although this reaction is neither inhibited nor stimulated by the presence of catalase, TCDO appears to act in a similar fashion to H_2O_2. Peroxidase can be replaced by hemoglobin (Sigma) but not by equimolar amounts of Fe^{2+} or Fe^{3+} [2].

2) Kinetics of activation and inactivation

In order to gain an impression of the stability of the TCDO-hemeprotein complex, TCDO was incubated with various activators for different time periods before the indicator compound was added. These preincubations were shaken or mixed in air.

As can be seen from Figure 1, 10^{-5}M Fe^{2+}-hemoglobin (Sigma) and 10 units POD show quite different effects. POD appears to have an activity half-life of about 30 to 40 min whereas hemoglobin shows an activity maximum after about 30 min preincubation. After this time, an inactivation phase begins which lasts for about 1.5 h.

After this time, no active species is present in the reaction mixture which is capable of oxidising KMB.

Further marked differences are observed when the effect of Fe^{2+}, Fe^{3+} -hemoglobin, myoglobin and blood on TCDO activation and inactivation are compared. Each catalyst was present at a concentration of 10^{-5}M heme. Hemoglobin shows a linear activation lasting about 10 min, followed by a similarly linear loss of activity. The half-life of the inactivation phase is about 30 min after maximum activity.

Myoglobin appears to act in a similar manner to POD. It requires no activation phase, but rather shows a plateau of maximum activity (5–10 min duration), followed by a slow deactivation with a half-life between 35 min and 50 min. Blood reacts in a different manner to both hemoglobin and myoglobin. At the start, the activity is virtually zero and remains low for the first 30 min of preincubation. The main activation phase begins after about 30 min and lasts for approximately 30–40 min before an activation maximum is reached (Figure 2).

A clear sigmoidal activation curve is obtained when the kinetics of blood-TCDO activation are followed over a time period of 3 h, which lasts about 60 min. The

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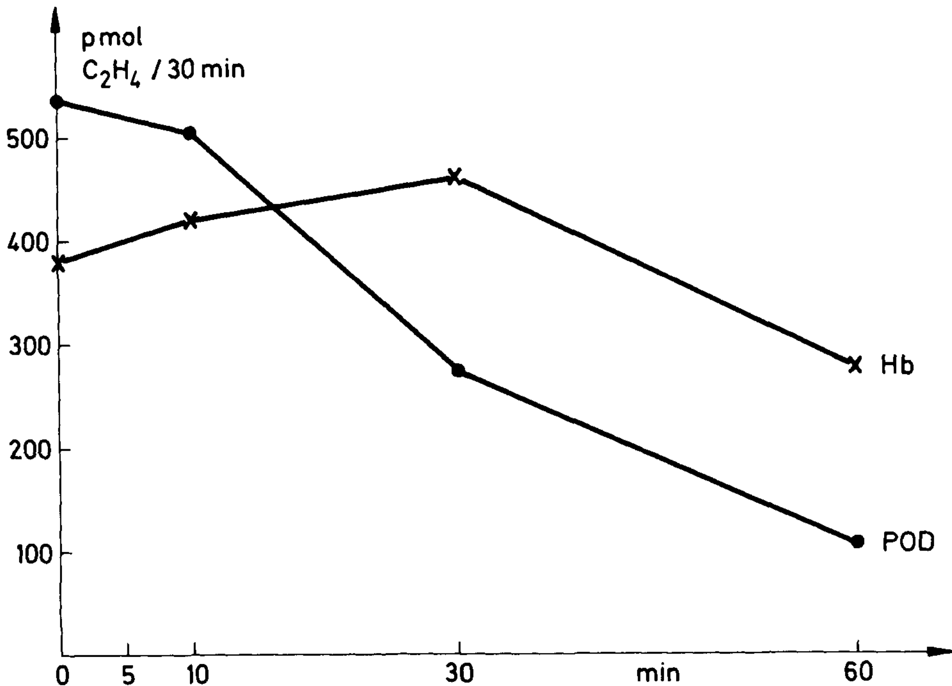

FIGURE 1 Time course of ethylene formation from KMB driven by TCDO and catalyzed by POD or Hb.
Reaction conditions: 2 ml 0.1 M phosphate buffer pH 7.8 contained: 10 U POD; 10^{-5}M Hb; 10 μl TCDO. Times indicated represent intervalls of preincubations without the indicator, KMB. After these times 50 μmol KMB was injected into the rubber-sealed vessels and reincubated at 25°C. After 30 min 1 ml gas from the headspace of the 14 ml Fernbach flasks was withdrawn and ethylene was determined gaschromatographically.

maximum activity is reached after 120 min, followed by an inactivation phase with a half-life of about 35 min (Figure 3). The maximum activation rates are observed between 30 min and 60 min preincubation and the highest inactivation rates are seen between 150 min and 180 min. Experiments in which blood and hemoglobin were mixed (blood/hemoglobin, 1:1) showed that the activation phase required by blood was not due to the inactivation of an inhibitor. Addition of increasing amounts of the detergent "Triton X-100" eliminates the blood activation phase as compared to incubation of TCDO with hemoglobin (Figure 4).

DISCUSSION

The question of the biochemical mechanism of the antibacterial action and the induction of increased oxygen supply of hypoxic, peripheral tissue by TCDO can be answered on the basis of the studies reported here in comparison with data from the literature.

1) The antibacterial action of TCDO[4,8], especially against anaerobes (e.g.

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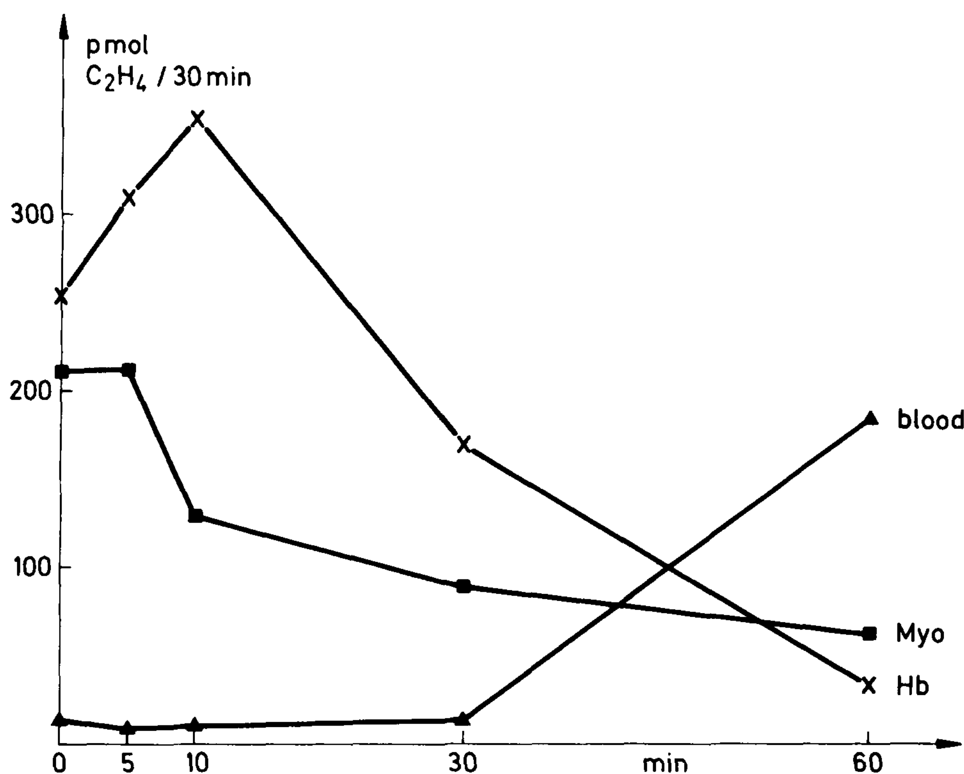

FIGURE 2 Time course of ethylene formation from KMB driven by TCDO and activated by Hb, Myo or blood.
Reaction conditions: See figure 1. Myo, blood, according to 10^{-5}M heme-iron.

Clostridium perfringens) appears to be due to the production of active oxygen species from the reaction of TCDO with hemoglobin or myoglobin. The oxidising ability of this new type of oxidant lies approximately between that of superoxide and the $OH^{\cdot}$ radical. It is comparable with the leucocyte-simulating 'Klebanoff system' which in a modified form, also appears to be responsible for part of the hypersensitive reaction and lignification in higher plants[9,10]. This system comprises POD, NADH, Mn^{2+} ions and is stimulated by monophenols. It catalyses the formation of ethylene from KMB, similarly to TCDO-hemoglobin, TCDO-POD, TCDO-myoglobin or xanthine/xanthine oxidase. Ethylene formation from methionine occurs via the agency of the $OH^{\cdot}$ radical or singlet oxygen, but not by xanthine oxidase or a TCDO-hemoprotein complex. An oxidant similar to the "Klebanoff system" appears to be responsible for the bactericidal action and the induction of accompanying reactions in inflammation by activated leucocytes in phagocytosis[11].

2) The initiation of increased oxygen supply in hypoxic tissue by TCDO is a complex process which may occur as follows: As can be seen in Figure 2, myoglobin reacts with TCDO without an activation phase (i.e. very fast), whereas blood requires a period of preincubation of about 45 min in order to achieve comparable rates. This is apparently due to slow penetration of TCDO into the erythrocytes and/or partial lysis

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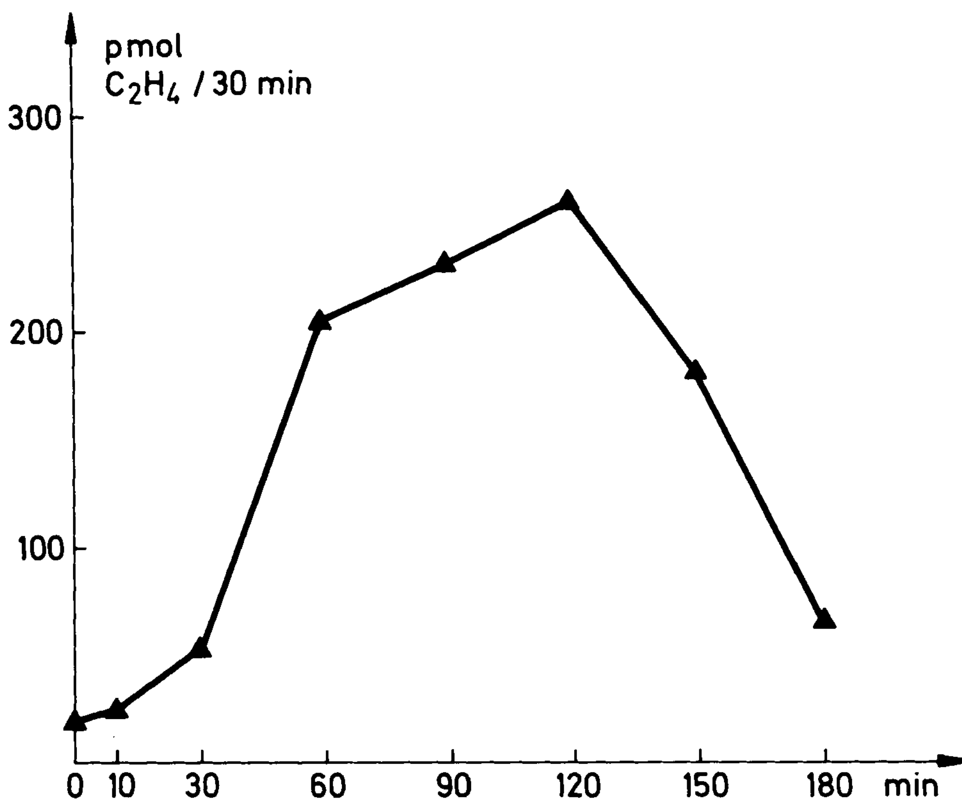

FIGURE 3 Time course of ethylene formation from KMB driven by TCDO and catalyzed by blood. *Reaction conditions:* See figures 1 and 2.

and thus Hb-release. In both cases, the product of the reaction is a biologically relevant oxidant which can be compared with the myeloperoxidase-catalysed reaction which, however, does not lead to the production of singlet oxygen (cf. 12).

For experimental purposes, the xanthine oxidase system is often used instead of the "Klebanoff system" or isolated leucocytes. In many respects, this enzymatic system reflects the situation in activated leucocytes, macrophages and monocytes in that many types of active oxygen species are simultaneously produced e.g. $O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$-like radicals and Fenton oxidants. It has been demonstrated that similar to activated leucocytes, xanthine oxidase possesses vasodilatory properties[5]. Myoglobin-TCDO and POD-TCDO possess activities similar to the "Klebanoff system" and appear to be comparable to the xanthine oxidase reaction in that KMB is oxidised, whereas methionine remains unaffected. These activities also occur under anaerobic conditions and thus the TCDO activation of heme can be summarised in the following scheme which is partly hypothetical. The times indicated refer to reactions in the *in vitro* system discussed here.

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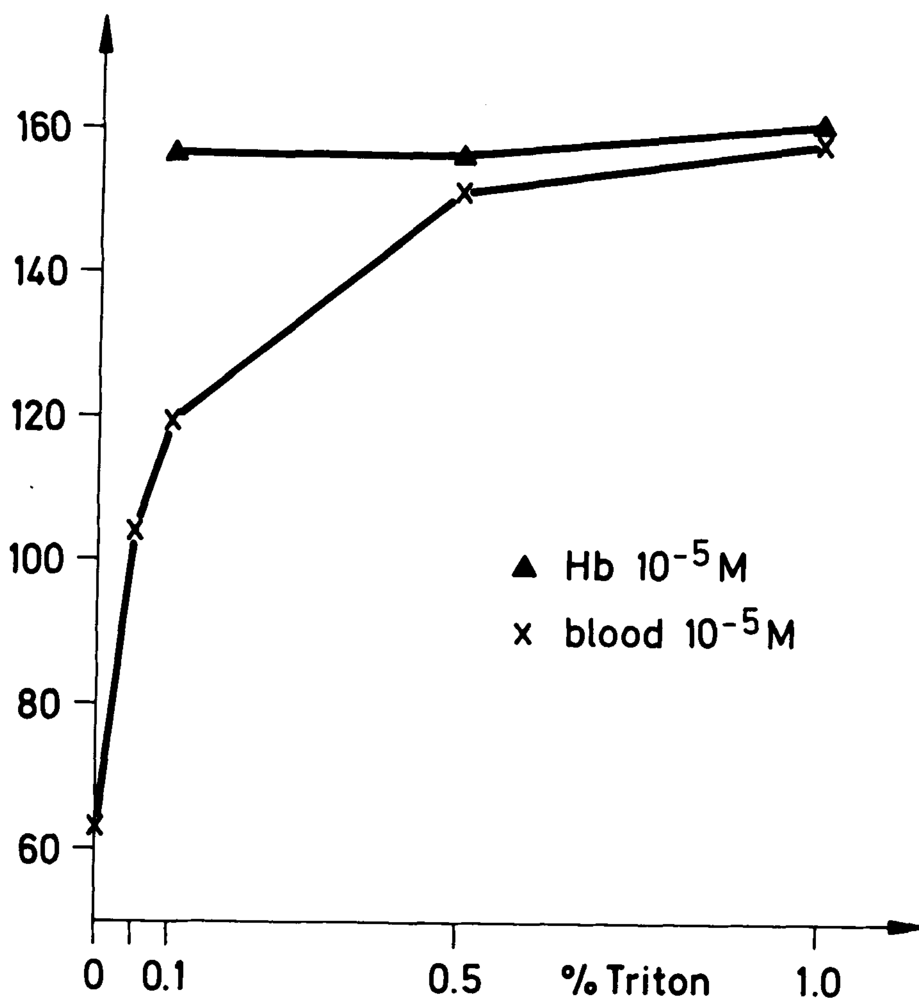

FIGURE 4 "Blood"-activation by TCDO in dependence of Triton X-100 as compared to isolated hemoglobin.
Reaction conditions: See figure 1. The indicated mixtures were incubated for 30 min with 50μ Mol KMB without preincubation.

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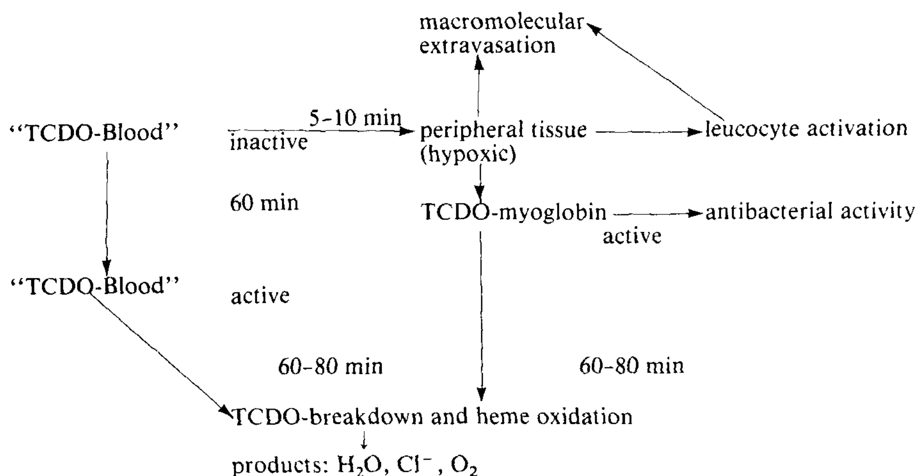

In contrast to xanthine oxidase in ischaemic processes[13-15] and activated leucocytes, the above reactions appear not to enhance the production of excessive OH$^\cdot$ radicals during the time-limited leucocyte activation. Therefore, it is unlikely that an uncontrolled generation of aggressive oxygen species occurs (cf. 16) and thus associated side reaction such as edema formation and other secondary reactions of inflammation[17] are unlikely to be a major problem in the use of OF.

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