Peroxisomal β -oxidation from endogenous substrates

Demonstration through H_2O_2 production in the unanaesthetized mouse

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A system was developed in which it is possible to detect *in vivo* changes in hepatic H_2O_2 production, using a combination of the catalase inhibitor, 3-amino-1,2,4-triazole and methanol. In mice, starvation significantly increases hepatic H_2O_2 production and plasma non-esterified fatty acid concentrations. Short-term refeeding after a 24 h starvation period brings H_2O_2 production and plasma non-esterified fatty acid concentration back to normal in 3 h. Administration of insulin 24 h after the onset of starvation normalizes H_2O_2 production in less than 2 h and decreases non-esterified fatty acid concentration below normal values. The suppression by insulin of H_2O_2 production, as well as its coherence with plasma non-esterified fatty acid concentration, indicate that increased H_2O_2 production in starved mice reflects peroxisomal β -oxidation.

In liver, fatty acid oxidation occurs in mitochondria and peroxisomes. Literature data on the relative importance of both systems, obtained largely from peroxisomal fractions and liver homogenates, seem to favour a preponderance of the mitochondrial β -oxidation system rather than the peroxisomal one. Mannaerts *et al.* (1979) claim that peroxisomal β -oxidation is of little (less than 10%) quantitative importance, on the basis of studies using rat liver homogenates and isolated hepatocytes. According to Foerster *et al.* (1981), the assayable capacity for peroxisomal β -oxidation in the intact isolated perfused rat liver is used to only a minor degree. Kondrup & Lazarow (1982) on the other hand, using a method that avoids the use of metabolic inhibitors, calculate the peroxisomal contribution to be 32% of total fatty acid oxidation in isolated rat liver hepatocytes. A few specific functions, such as the oxidation of very-long-chain and mono-unsaturated fatty acids, have been ascribed to the peroxisomal system (Christiansen *et al.*, 1978; Osmundsen & Neat, 1979; Neat *et al.*, 1981; Kawamura *et al.*, 1981; Bremer & Norum, 1982). A number of physiological conditions are known in which the enzymic capacity of the peroxisomal β -oxidation system is elevated. This is

the case when rats are fed high-fat diets (Neat *et al.*, 1980), in starved rats (Ishii *et al.*, 1980) and in genetically obese mice (Murphy *et al.*, 1979). In all these conditions there is an elevated fatty acid supply. The enzyme activities of peroxisomal β -oxidation are also elevated considerably in the livers of rats fed hypolipidaemic drugs (Lazarow, 1980). No direct measurements of fatty acid fluxes through the two oxidation pathways are as yet available *in vivo*.

In both the mitochondrial and the peroxisomal fatty acid oxidation the four intermediate reaction products are the same. The enzymes catalysing the reactions however are all different. The first enzyme in peroxisomal β -oxidation is a flavoprotein oxidase, with FAD as its prosthetic group. The enzyme transfers two electrons from an acyl-CoA to molecular oxygen, with the formation of an enoyl-CoA and H_2O_2 . Increased activity of this oxidase will be accompanied by increased production of H_2O_2 . Evaluation of H_2O_2 production *in vivo* could be a powerful means of examining the enzymic activity of the peroxisomal system. Inhibition of the catalase- H_2O_2 complex (compound I) by aminotriazole is dependent on H_2O_2 production (Margoliash *et al.*, 1960). Measurement of the residual catalase activity (RCA) after administration of aminotriazole permits evaluation of H_2O_2 production *in vivo* (Geerts & Roels, 1982).

Abbreviations used: aminotriazole, 3-amino-1,2,4-triazole; RCA, residual catalase activity.

In our experimental set-up compound I is in part decomposed by methanol (Oshino *et al.*, 1973; Jones *et al.*, 1978). Methanol lowers the concentration of compound I to a new steady state, and allows detection of changes in the compound I concentration, which could otherwise pass undetected due to maximal saturation of catalase (Oshino *et al.*, 1975a). A lower concentration of compound I will result in a diminished inhibition by aminotriazole (Margoliash *et al.*, 1960). When in the methanol-treated system H_2O_2 production is stimulated by exogenous substrates, compound I rises again (Oshino *et al.*, 1973; Jones *et al.*, 1978) and as a consequence aminotriazole inhibition will increase. Methanol is to be preferred as a hydrogen donor in this system. Indeed the oxidation of methanol occurs almost exclusively through the peroxidative system, whereas ethanol utilizes both the peroxidative and the alcohol dehydrogenase pathways in rats, mice and guinea pigs (Tephly *et al.*, 1964; Harris *et al.*, 1982).

The present study investigates whether H_2O_2 production *in vivo* from endogenous substrates in mice is elevated in conditions that are known to induce an increased oxidation of fatty acids. A well-known physiological situation affecting plasma non-esterified fatty acid concentration and β -oxidation is starvation (Bremer, 1981; McGarry & Foster, 1981; Müller *et al.*, 1982; Saggerson, 1982; Laker & Mayes, 1982; Bradley & McDonald, 1982; Royle *et al.*, 1982; Rémésy & Demigné, 1983). During a 24 h starvation period the concentrations of glycogen in the liver and of insulin and glucose in plasma decrease, whereas the concentrations of plasma ketones and plasma non-esterified fatty acid increase considerably (McGarry *et al.*, 1973). We also investigate the effect of short-term refeeding on H_2O_2 production *in vivo*. Refeeding causes a brisk rise in plasma glucose and plasma insulin concentrations; plasma ketones and non-esterified fatty acid concentrations on the contrary show a prompt decrease (McGarry *et al.*, 1973; Seitz *et al.*, 1977). Insulin is a potent antilipolytic agent and a stimulator of hepatic lipogenesis, resulting in decreased non-esterified fatty acid release; it also suppresses fatty acid oxidation in the liver (Lopez *et al.*, 1959; Campbell & Green, 1966; Raben & Hollenberg, 1960; Gorden & Cherkas, 1960; Bieberdorf *et al.*, 1970; McGarry & Foster, 1980; Beynen *et al.*, 1980; Beynen, 1982). For this reason we also examine the effect of insulin on H_2O_2 production in starved mice. Preliminary data have been published (Kerckaert *et al.*, 1982; Van den Branden *et al.*, 1983).

Materials and methods

Male BALB-C mice (25–30 g) were used in our experiments. Aminotriazole (1 g/kg in 0.9% NaCl),

uric acid (0.17 g/kg in 0.5% Li_2CO_3 , with the pH adjusted to 7.2), glycollic acid (0.50 g/kg in 0.9% NaCl, with the pH adjusted to 7.5) and methanol (3.2 mmol/kg) were administered by intraperitoneal injection. Insulin (1 i.u./kg in 0.9% NaCl; Insulin Novo Actrapid MC) was given by intramuscular injection. In order to study the inhibition of catalase by aminotriazole as a function of time, animals were killed (dislocation of the neck) 15, 30, 60 and 120 min after aminotriazole administration. In all other experiments inhibition time by aminotriazole was 1 h. In most experiments aminotriazole and methanol were administered simultaneously.

Fed mice received standard laboratory diet (A03-UAR France). Starved mice were deprived of food (water *ad libitum*) for 24 h. Refed mice received the standard diet together with drinking water containing 100 g of glucose/l during 1, 2 and 3 h after a 24 h starvation period. Insulin-treated mice received an insulin injection 1 h before administration of aminotriazole and methanol.

Catalase activity was assayed at 0°C in the total liver homogenate by the titanium oxysulphate method (Baudhuin, 1974). 1 U_B is the amount of catalase which breaks down 90% of the substrate (1.5 mmol/l H_2O_2) in a volume of 50 ml at 0°C in 1 min; maximal reaction time is 10 min. For each animal the catalase activity calculated is the mean of ten measurements at ten different reaction times. Residual catalase activity (RCA) is the catalase activity that remains after inhibition by aminotriazole alone or in combination with methanol.

Ketone bodies (3-hydroxybutyrate and acetoacetate) in mouse liver were assayed according to Williamson *et al.* (1967).

Free fatty acid concentrations in plasma were determined by the colorimetric micromethod of Falholt *et al.* (1973), modified by Blum *et al.* (1982).

Uric acid determination in plasma was done according to Praetorius & Poulsen (1953).

Each experimental group consisted of at least five animals. All results are presented as the mean $\pm$ S.E.M. For statistical analysis, the Mann-Whitney test was used (Mann & Whitney, 1947).

Results

Catalase activity in the liver of untreated, standard diet fed mice is $88.68 \pm 2.48 U_B/g$ wet liver. After administration of aminotriazole, RCA declines exponentially with time (Fig. 1), reaching a value of $12.07 \pm 0.80 U_B/g$ of liver after 30 min. This value does not significantly diminish at 60 and 120 min. Because a stable value of RCA is certainly reached after 1 h, we chose a 1 h period for aminotriazole inhibition in all the following

Fig. 1. Residual catalase activity (RCA) in total mouse liver homogenate after a single injection of aminotriazole. Animals were killed 15, 30, 60 and 120 min after intraperitoneal injection of aminotriazole (1 g/kg). Each point represents the mean $\pm$ S.E.M., obtained for the number of animals indicated in parentheses.

experiments. Simultaneous administration of aminotriazole and methanol causes RCA to rise from 12.74 ± 2.65 U_B/g of liver to 47.55 ± 4.10 U_B/g of liver 1 h after injection. The same methanol concentration (3.2 mmol/kg) was used in all further experiments.

To test the efficiency of our method in detecting increased H₂O₂ production *in vivo*, we injected animals with urate and glycolate, two substrates of peroxisomal H₂O₂-producing oxidases, which are known to cause a rise of compound I in rats (Oshino *et al.*, 1975a). The results in Table 1 show that RCA values decrease significantly following urate and glycolate administration.

The effects on RCA of starvation, refeeding after starvation, and insulin administration combined with starvation are summarized in Table 2. Starvation for 24 h causes a significant ($P < 0.01$) lowering of RCA as compared with the control group of fed animals. When only aminotriazole is administered without methanol, the difference between fed and starved animals is still significant. This excludes the role of methanol metabolism by pathways other than catalase. Refeeding and administration of insulin cause a return to normal values of RCA as found in the control group.

Neither administration of methanol and insulin alone nor starvation alone significantly influence catalase activity values in our experimental animals (Table 3).

Table 1. Residual catalase activity (RCA) in total mouse liver homogenate after addition of urate and glycolate. All mice received aminotriazole (1 g/kg) and methanol (3.2 mmol/kg) intraperitoneally for 1 h. Both urate (0.17 g/kg, intraperitoneally, 1 h) and glycolate (0.50 g/kg, intraperitoneally, 1 h) treated groups are significantly different from the control group ($P < 0.01$).

Conditions	RCA (U _B /g of liver)
Fed	$47.55 \pm 4.10(10)$
Fed + uric acid	$20.65 \pm 3.59(5)$
Fed + glycollic acid	$20.66 \pm 2.40(5)$

Table 2. Residual catalase activity (RCA) values in total mouse liver homogenate under different feeding conditions and after insulin administration

Mice received aminotriazole (1 g/kg) and methanol (3.2 mmol/kg) or aminotriazole alone intraperitoneally for 1 h. Fed mice received standard laboratory diet. Starved mice were deprived of food for 24 h. Refed mice received standard diet + 100 g of glucose/l in the drinking water 1, 2 and 3 h before killing. Insulin-treated mice received 1 i.u. of insulin/kg intramuscularly 2 h before killing. Statistical significance of differences: (a) versus (b), (a) versus (c), (b) versus (d), (b) versus (e): $P < 0.01$; (b) versus (c), (a) versus (d), (h) versus (i): $P < 0.05$.

Conditions	RCA (U _B /g of liver)	
	Aminotriazole + methanol	Aminotriazole alone
Fed	$47.55 \pm 4.10(10)$ (a)	$12.74 \pm 2.65(8)$ (h)
Starved	$20.99 \pm 1.53(5)$ (b)	$4.30 \pm 0.84(5)$ (i)
Refed, 1 h	$30.00 \pm 2.16(5)$ (c)	
Refed, 2 h	$43.20 \pm 5.92(5)$ (d)	
Refed, 3 h	$46.82 \pm 3.59(5)$ (e)	
Fed + insulin	$39.90 \pm 0.73(6)$ (f)	
Starved + insulin	$56.05 \pm 3.03(5)$ (g)	

Table 3. Catalase activity in total mouse liver homogenate after addition of methanol or insulin, and after fasting. Catalase activity was determined after injection of methanol alone (3.2 mmol/kg, 1 h, intraperitoneally) or insulin alone (1 i.u./kg, 2 h, intramuscularly) and after starvation (24 h) alone. There is no significant difference between the four groups.

Conditions	Catalase activity (U _B /g of liver)
Fed	$88.68 \pm 2.48(13)$
Fed + methanol	$74.75 \pm 7.95(5)$
Fed + insulin	$77.33 \pm 7.93(5)$
Starved	$85.58 \pm 5.19(6)$

During the starvation period of 24 h plasma non-esterified fatty acid concentration increases. Refeeding and insulin administration lower plasma non-esterified fatty acid content (Table 4).

Table 4. Plasma non-esterified fatty acid concentrations in mice in different feeding conditions and after insulin administration

Fed mice received standard laboratory diet. Fasted mice were deprived of food 24h before killing. Refed mice received standard diet + glucose (100 g/l) in the drinking water 3h before killing. Insulin-treated mice were injected with insulin (1 i.u./kg) intramuscularly 2h before killing. Statistical significance of differences: (a) versus (b), (a) versus (d), (b) versus (c), (b) versus (d), (c) versus (d): $P < 0.01$.

Conditions	Plasma non-esterified fatty acids (mmol/l)
Fed	$0.40 \pm 0.09(5)$ (a)
Starved	$1.18 \pm 0.12(5)$ (b)
Refed 3h	$0.61 \pm 0.15(5)$ (c)
Starved + insulin	$0.18 \pm 0.04(5)$ (d)

Liver concentrations of acetoacetate and 3-hydroxybutyrate are 0.141 ± 0.020 and $0.385 \pm 0.036 \mu\text{mol/g}$ of liver in fed animals, and increase to 0.242 ± 0.030 and $1.327 \pm 0.192 \mu\text{mol/g}$ of liver in starved animals.

Discussion

After 1h of inhibition with aminotriazole, RCA is 14% of the basic value, with a small s.e.m. This is in contrast with the curve found for the guinea pig, where it takes 4h to reach relatively homogeneous values for all animals (Geerts & Roels, 1982).

The efficiency of the method to detect increases in H_2O_2 production *in vivo* is proven by the urate and glycollate experiments. Urate and glycollate are substrates for the H_2O_2 -producing enzymes uric acid oxidase and hydroxyacid oxidase respectively, both located in the peroxisomes (De Duve & Baudhuin, 1966). In perfused rat liver, administration of these substrates results in higher H_2O_2 production (Oshino *et al.*, 1975a) and this is reflected by a higher inhibition of catalase in the aminotriazole/methanol system.

Compared with the control group of normally fed animals, starvation results in a significant lowering of RCA. This leads us to conclude that starvation causes an increased production of H_2O_2 by the liver. However, after 24h of starvation, we noted a 10% decrease in liver volume and a 15% decrease in liver weight. This liver-shrinking effect of starvation has also been reported in the literature (Uhal & Roehrig, 1982; Saggerson, 1982; McGarry & Foster, 1982). Catalase activity per gram is the same in fed and starved animals. We therefore must conclude that total liver catalase is diminished, a fact also reported by Rechcigl & Price (1961). In case oxidases are not diminished to the same degree, and their H_2O_2 production

remains unchanged during starvation, this might lead to a lowering of RCA not caused by elevated H_2O_2 production, but by the increase of oxidases relative to catalase activity. For this reason we investigated whether reversal of increased H_2O_2 production could be obtained by experiments of very short term, which excludes significant enzyme induction or breakdown. Indeed, many of the regulatory enzymes of hepatic carbohydrate and lipid metabolism have half-lives of 24h or more (Beynen, 1982). If increased formation of compound I during starvation is not linked to an increased flux of fatty acids through the fatty acid oxidase, we do not expect any change in RCA after short-term refeeding or short-term administration of insulin. Yet we demonstrate a significant fall in inhibition by aminotriazole in the first hour after the start of refeeding. This decrease continues, until the value found in normally fed animals is reached after 3h. Insulin administration 24h after the onset of starvation brings the RCA value back to normal in less than 2h. These results prove that increased H_2O_2 production during fasting is a reality and not an artifact due to a decrease in total liver catalase.

Summarizing our main results, we may state that hepatic H_2O_2 production is modified by those experimental conditions that are known to raise or decrease the flux of fatty acids through the oxidation pathways (i.e. starvation, refeeding and insulin administration). We conclude therefore that the flux of endogenous substrate through the peroxisomal fatty acid oxidase is responsible for the observed changes in H_2O_2 production.

As expected, plasma non-esterified fatty acids are raised during starvation, and are normalized by refeeding or insulin administration. These changes occur in parallel with hepatic H_2O_2 production. Our results do not show whether the plasma non-esterified fatty acid concentration is an independent factor controlling peroxisomal β -oxidation. A direct influence of insulin on peroxisomal β -oxidation is not excluded. Total, i.e. mitochondrial plus peroxisomal, fatty acid oxidation is suppressed by insulin in the presence of elevated levels of non-esterified fatty acid (Bieberdorf *et al.*, 1970).

Our observations reflect an increased flux of substrate through the β -oxidation pathway, and not necessarily an increase in enzyme specific activity. Ishii *et al.* (1980) have demonstrated a 2.2-fold rise in cyanide-insensitive palmitoyl-CoA oxidizing activity after 1 day of starvation. The question cannot be answered whether similar changes would suffice to explain the reversal at very short term of H_2O_2 production that we report in this paper.

Is it possible that increased H_2O_2 production during starvation is due to a peroxide source other

than increased activity of peroxisomal β -oxidation? Several H_2O_2 -producing oxidases other than the fatty acid oxidase are present in peroxisomes. De Duve & Baudhuin (1966) demonstrated the presence of uric acid oxidase, hydroxyacid oxidase and D-amino acid oxidase. An increased urate metabolism during starvation is unlikely, as we found plasma urate concentration to be unchanged in fed and fasted mice. D-Amino acids, if present, must originate from intestinal bacteria, since vertebrates do not possess D-amino acids. If starvation provokes any change, a decrease would be expected. Ishii *et al.* (1980) report a decrease of urate oxidase and D-amino acid oxidase during the first 24 h of starvation. Hydroxyacid oxidase acts on glycolate and lactate. Glycolate metabolism is not known to be very important in animals. In fasting rats lactate becomes the main glucogenic substrate (Rémésy & Demigné, 1983). In adult humans, lactate concentration in blood is significantly decreased after 6 h of fasting and remains at a low level during an 84 h fasting period (Haymond *et al.*, 1982).

In the cell, competition for H_2O_2 generated exists between the catalase and glutathione peroxidase pathways (Isaacs & Binkley, 1977). Fasting causes a decrease of hepatic reduced glutathione (GSH) in rats (Harisch, 1982) and mice (Brooks & Pong, 1981). However, this does not cause a decrease of H_2O_2 breakdown by the GSH-oxidase pathway in liver homogenates, probably because after 24 h starvation the GSH concentration is still fairly high and H_2O_2 production is the rate-limiting factor rather than GSH concentration (Isaacs & Binkley, 1977). The same authors also report that the GSH pathway is favoured in starvation.

Several authors investigated peroxisomal β -oxidation in isolated hepatocytes and perfused liver. Foerster *et al.* (1981) demonstrate an increased H_2O_2 production in the intact isolated perfused rat liver on infusion of exogenous fatty acids such as octanoate, decanoate, laurate, myristate and erucate. The rate increases with chain length. Isaacs & Binkley (1977) find that in liver homogenates of starved rats, endogenous H_2O_2 production is increased, which agrees with our results.

According to Mannaerts *et al.* (1979) peroxisomal fatty acid oxidation is not enhanced in starvation. In their experiments *in vitro*, the same concentration of palmitoyl-CoA was given to liver homogenates prepared from fed or starved rats, a situation quite different from the living animal. Data concerning mitochondrial β -oxidation in starved rats under the same experimental conditions are not given.

Oshino *et al.* (1975b) describe a decrease of H_2O_2

production in the livers of starved rats. However, their measurements were done on livers perfused with a buffer solution containing lactate and pyruvate, a condition that is very unlike starvation, where carbohydrate is decreased.

In our experiments the striking coherence between changes in H_2O_2 production and plasma non-esterified fatty acids, on the one hand, and the immediate suppression of H_2O_2 production, by insulin or refeeding, on the other, are evidence for the functioning of the peroxisomal fatty acid oxidase in starved animals.

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