Effect of Metal Ions on the Formation and Destruction of Hydrogen Peroxide in Media used for Mammalian Adipocytes Incubation

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Summary: The activity of transition metals under incubation conditions is of particular interest because these metal ions induced an insulin-like effect in fat cells which is reminiscent of that of hydrogen peroxide. It is possible that in one of its bound states nickel may stimulate hydrogen or lipid peroxide accumulation which in turn can account for this effect on adipocytes during in vitro incubation. In this study using a simple model system the role of metal ions on the formation and destruction of hydrogen peroxide is illustrated in medium used for mammalian adipocytes incubation. It is interesting to note that the only metal which generated hydrogen peroxide in the medium was copper when its concentration was 10 μM. The metals associated with destruction of hydrogen peroxide were cobalt, copper and manganese. Iron and nickel played no role at all.

Introduction

Considerable advances have been made in elucidating the mechanisms involved in the formation and degradation of lipid peroxides in living tissues. The accumulation of lipid peroxides is governed by the balance between the processes responsible for their synthesis and degradation. Although both processes are subject to variations, the lipid peroxide level in the tissues of intact animals has generally been found to remain within narrow limits. However, the situation that arises in tissues incubated in vitro is considerably more variable. Not only are there gross changes in the availability of the substrates, the polyunsaturated fatty acids and molecular oxygen, another key factor is the composition of incubation medium.

The situation is well illustrated in the case of adipose tissue: a number of workers have found that its response to added reagents was due to the hydrogen peroxide generated in the incubation system. Raben and Mutsuzaki [1] proposed that the purines inhibited catecholamine induced lipolysis by oxidizing the hormone to its quinone derivative. Xanthane oxidase, which is known to generate hydrogen peroxide during the oxidation of purine and other substrates [2], has been shown to be

present in adipose tissue and isolated fat cells [3]. Again, thiol compounds have been shown to have metabolic activities in fat cells which are due to the generation of hydrogen peroxide by their interaction with Cu^{2+} ions present in trace levels in the incubation medium used [4]:

$$2\ RSH + Cu^{2+} = RSSR + Cu^{+} + 2H^{+} \quad (i)$$
$$2\ H^{+} + 2\ Cu^{+} + O_2 = Cu^{2+} + H_2O_2 \quad (ii)$$

Yet another example arises in connection with studies on the actions of polyamines [5]. The observed effects were shown to be due to hydrogen peroxide generated by a polyamine oxidase present in the incubation system.

Considerable interest has been taken in the metabolic effects of hydrogen peroxide which have been characterized as insulin-like [6]. Its effects on glucose metabolism and pyruvate dehydrogenase activity have been investigated in some detail [7 and 8]. Hydrogen peroxide tends to promote lipid peroxidation. The effects of these products on adipose tissue function are not well established. Nevertheless, they have a considerable membrane-disruptive action which might be expected to have a generally depressive effect on the tissue. They may also influence metabolism through their stimulatory effect on ion permeability [9], prostaglandin synthesis [10], and the alteration of thiol levels [11]. All these changes have been presented as possible causes of the correlation between lipid peroxidation and cell proliferation.

Results and Discussion

A number of miscellaneous observations have been made that media used for the incubation of adipose tissue and isolated adipocytes generate hydrogen peroxide under certain circumstances. A survey of the conditions that influence the rates of both the formation and destruction of this substance was therefore undertaken, using a method capable of detecting μmolar quantities.

In several experiments, hydrogen peroxide was determined in Kreb's Ringer bicarbonate buffer after various periods in the presence of metal ions, both alone and in combination with cysteine (adipose tissue or isolated adipocytes were not present). Calibration curves showed that cysteine considerably decreased the colour produced by hydrogen peroxide (approximately 30% at the cysteine concentration used) unless an excess of N-ethylmaleimide was added (see methods). A typical result is shown in Fig. 1 in which samples of incubation medium containing Cu^{2+} (10 μM) and cysteine (1 mM) were removed for analysis after 0, 30, 60 and 90 minutes. Whereas neither reagent caused hydrogen peroxide accumulation when added alone, in combination they reacted rapidly but over a relatively short period; little hydrogen peroxide accumulation occurred after 1 h of incubation. The concentration of Cu^{2+} used appears to be critical; further experiments showed that in the presence of higher concentration of Cu^{2+} (100 μM), hydrogen peroxide accumulation both in the presence and absence of cysteine was prevented (the results, which showed that the higher concentration of Cu^{2+} did not affect the hydrogen calibration curve, are not shown).

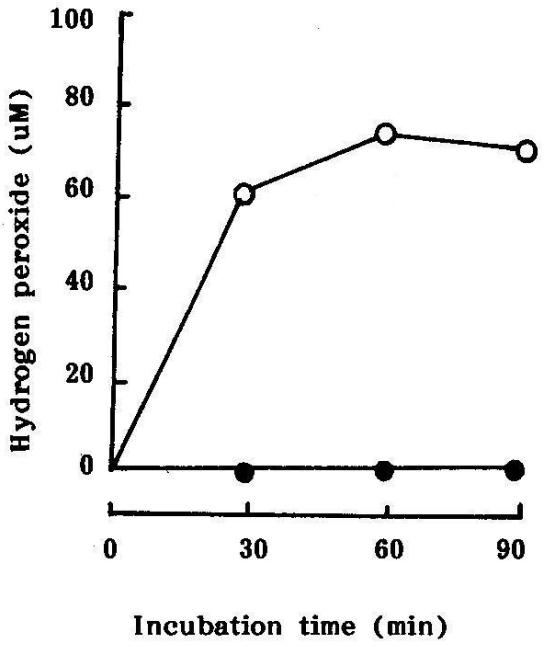

Fig. 1: The effect of copper ions on formation of hydrogen peroxide in cell-free incubation medium containing cysteine.

The hydrogen peroxide content was determined in the samples obtained at different intervals during incubation at ambient temperature from (•–•) Krebs Ringer phosphate buffer containing (•–•) cysteine (1mM) and (•–•) Cu^{2+} (10 μM) alone or (0–0) in combination.

The present study was undertaken principally in order to determine the specificity of this reaction in terms of its metal ions requirement. Progress

curves for hydrogen peroxide formation in both the presence and absence of cysteine (1 mM) were determined under identical conditions except that Cu^{2+} was replaced at both 10 and 100 μM concentrations by other metal ions. Under no condition could the Cu^{2+} be mimicked by Fe^{3+}, Mn^{2+}, Co^{2+} or Ni^{2+} (results not shown).

When bovine serum albumin (1%) was added to the incubation medium, H_2O_2 accumulation due to Cu^{2+} (10 μM) and cysteine (1 mM) was eliminated. It would appear therefore that the H_2O_2 formation detected by Czech *et al.* [4] in the presence of Cu^{2+} (30 μM), cysteine (1 mM) and albumin occurred because of the low concentration of the latter (0.1%).

The H_2O_2 accumulation observed in the above experiments could be regarded as a measure of the rate of formation of this substance only if the H_2O_2 generated was stable in this medium. Further experiments were therefore undertaken to determine whether added H_2O_2 disappeared from the medium over a period of 60 minutes at 37°C. The results obtained are shown in Fig. 2. The addition of metal ions (10 μM; Fe^{3+}, Ni^{2+}, Co^{2+} and Mn^{2+}) had no effect on the H_2O_2 content of the medium irrespective of whether cysteine was present, except that in the presence of 10 μM Cu^{2+} and cysteine a further accumulation of H_2O_2 occurred. The same conclusion could be drawn when metal ion concentrations as high as 1 mM were used. Fig. 3 shows that H_2O_2 was stable in the presence of the higher concentration of Fe^{3+} and Ni^{2+} but not Co^{2+}, Cu^{2+} and Mn^{2+} irrespective whether cysteine and albumin were present or not.

Whereas hydrogen peroxide was found to be stable in media that had not been exposed to adipose tissue, the latter contained and released an enzyme which caused its rapid degradation. In a series of experiments, isolated adipocytes were incubated in Kreb's Ringer bicarbonate buffer containing bovine serum albumin (2%) for 15 minutes before an aliquot of the medium was tested for its activity in degrading added H_2O_2 (120 μM). The bulk of the H_2O_2 was degraded after a further 5 minutes of incubation; the extent being somewhat increased when the bovine serum albumin was saturated with exogenous linolenic acid (molar ration, 8:1) as shown in Fig. 4.

The activity of transition metals under incubation conditions used were particular intrest because

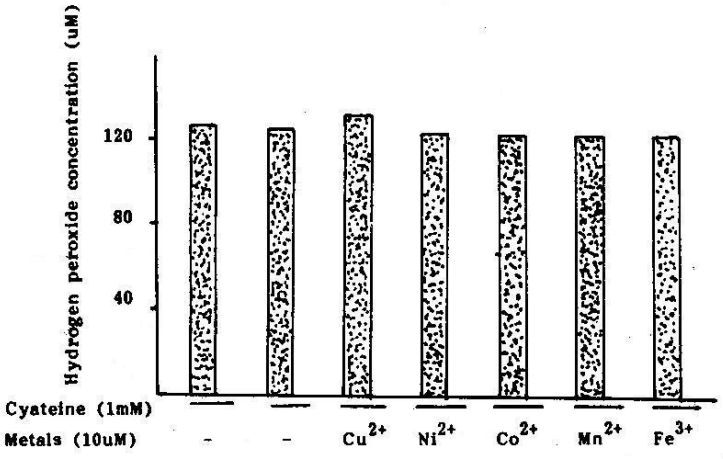

Fig. 2: The effect of low concentration of metal ions and cysteine on the exogenous hydrogen peroxide in the tissue-free Krebs Ringer phosphate medium.

The hydrogen peroxide content of medium containing exogenous hydrogen peroxide was determined after 1 hour of incubation at ambient temperature in the presence of cysteine (1mM) and metal ions (10 μM each). N-ethylmaleimide (0.1M) was added in assay. Each value is the average of two measurements.

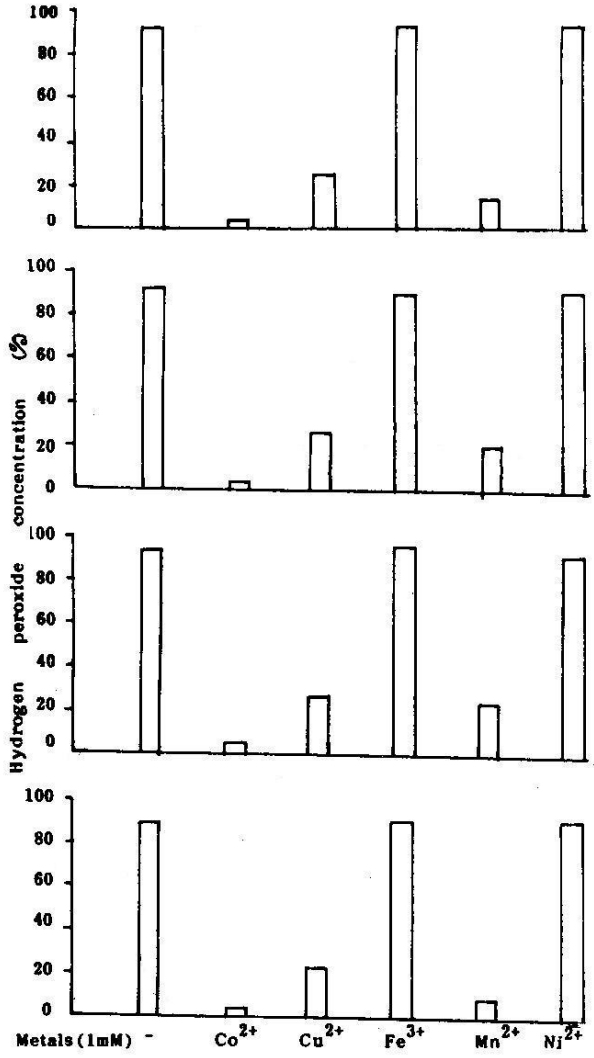

Fig. 3: The effect of high concentration of various metal ions on the stability of exogenous hydrogen peroxide in tissue-free medium containing cysteine and albumin.

Hydrogen peroxide was determined after an incubation period of 1h at ambient temperature in the presence of N-ethylmaleimide (2mM). Various metal ions (1mM each) were added to (a) Krebs Ringer bicarbonate medium with exogeneous hydrogen peroxide containing (b) cysteine (1mM) or (c) albumin (1%) alone or (d) in combination.

Saggerson *et al* [16] showed that these metal ions have an insulin-like effect in fat cells which is reminiscent of that of hydrogen peroxide [5]. It is possible that in one of its bound states Ni^{2+} might stimulate hydrogen peroxide or lipid peroxide accumulation which, in turn, could account for this effect. In this study, no evidence for such activity was detected. The hypothesis offered by Saggerson *et al.* [13] must therefore continue to be favoured; that the activity of transition metals is due to their influence on calcium metabolism.

The action of metal ions or hydrogen peroxide and lipid peroxide metabolism differed in several respects. This may not entirely surprizing since hydrogen peroxide in not precursor of lipid peroxide (Table 1 confirmed that the addition of hydrogen peroxide did not promote the formation of lipid peroxide in isolated adipocytes during *in vitro* incubation) except though it interacts with superoxide, ascorbate, etc., to produce hydroxyl radical and singlet oxygen [17]. Since hydrogen peroxide and lipid peroxide metabolism share certain enzymes, it is of interest that the hydrogen peroxide is rapidly degraded by the enzymes derived from adipose tissues (see Fig. 4).

Experimental

All the reagents employed in this research were of analytical grade. The Kreb's Ringer bicarbonate buffer as an incubating medium was prepared by the technique of Umbreit *et al.* [12]. The adipocytes from epididymal fat pads of albino rat were obtained according to method of Rodbell [13]. The cell-free medium was obtained after incubating isolated adipocytes for 15 minutes at 37°C in Kreb's Ringer bicarbonate buffer containing bovine serum albumin. The lipid peroxide was determined by the TBA-method of Sinnhuber and Yu [14].

Determination of Hydrogen Peroxide

The content of hydrogen peroxide was determined by the method of Trinder [15]. It is based on the following equation:

$$\text{chromogen} \xrightarrow[\text{peroxidase}]{H_2O_2} \text{coloured product}$$

a) Reagents

1. Phenol stock reagent was made by dissolving 1 g phenol (Analar) per litre of water.

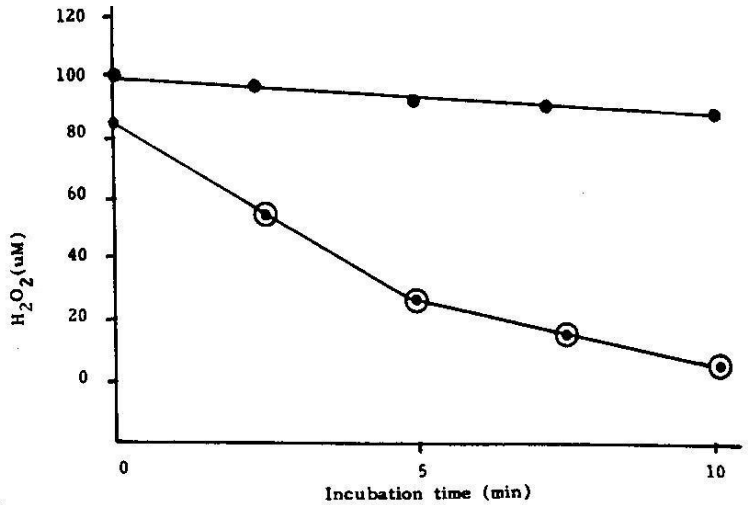

Fig.4: Loss of exogenous hydrogen peroxide in the cell-free medium obtained from pre-incubated adipocytes.

Adipocytes (10 mg triacylglycerol/ml) were incubated for 15 min at 37° in Krebs Ringer bicarbonate containing linolenic acid and albumin (molar ratio 8:1). Hydrogen peroxide added to the cell- free medium and determined at different times of incubation (o) at ambient temperature. An appropriate control (o) was run in parallel.

2. Colour reagent was made by dissolving 100 mg of 4- aminophenazone (BDH, UK) in 100 ml of phosphate buffer.

3. Peroxidase solution was made by dissolving 20 mg of horse-radish peroxidase (EC 1. 11. 1.7; Sigma Chemicals, USA) in 100 ml of phosphate buffer.

4. Phosphate buffer was made by dissolving KH_2PO_4 (5.95 g) and K_2HPO_4 (1.07 g) in 500 ml of water.

Assay

To the cuvette containing phenol reagent (0.1 ml), colour reagent (2.0 ml) and peroxidase (0.2 ml), the hydrogen peroxide-containing sample (1.0 ml) was added, thereafter, the accumulation of chromophore was determined at 515 nm using a Pye-Unicam SP-8000 spectrophotometer. The colour intensity slowly declined after reaching a maximum within 1 minute. The maximum value was, therefore, recorded. Where thiols (cysteine etc.) were present, an excess of N-ethylmaleimide (Koch-Light, UK) was added to eliminate their interference with colour formation [4].

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