Possible role of free radicals generated by pseudohypoxia in the regulation of hepatic glucose output. An in vitro model using rat liver microsomal glucose 6-phosphatase

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Summary Hepatic glucose output is decreased by hyperglycaemia through an unknown mechanism. We hypothesize that free radicals generated by hyperglycaemic pseudohypoxia might cause glucose output to decrease by inhibiting glucose 6-phosphatase – a key enzyme of gluconeogenesis. To prove this a model experiment was performed on a microsome fraction of rat liver. One of the characteristic features of pseudohypoxia due to hyperglycaemia is an increase in the ratio of NADH/NAD⁺, so in the present study the changes in NADH – induced glucose 6-phosphatase activity were investigated as related to the release of inorganic phosphate (Pi) derived from glucose 6-phosphate. After incubation for 50 min, Pi release was significantly reduced by NADH (4.026 ± 0.189 vs 2.696 ± 0.429 $\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{mg protein}^{-1}$, control vs NADH samples, $p < 0.01$). The decrease in the activity of glucose 6-phosphatase generated by NADH was prevented by using desferrioxamine, an irreversible

ferric chelator, butylated hydroxytoluene and Trolox, two agents which inhibit lipid peroxidation, and reduced glutathione, a non-specific radical scavenger. Superoxide dismutase, catalase and the hydroxyl radical scavenger dimethyl sulphoxide proved to be ineffective. When the above investigations were carried out in the presence of a ferric-EDTA complex the inhibition of glucose 6-phosphatase was found to be inducible by hydrogen peroxide and/or hydroxyl free radicals. These investigations seem to indicate that pseudohypoxia due to hyperglycaemia can inhibit the activity of glucose 6-phosphatase both by lipid peroxidation and by inducing hydrogen peroxide and/or hydroxyl free radicals and thus it may play a part in the glucose-induced decrease of hepatic glucose output. [Diabetologia (1997) 40: 1251–1254]

Keywords Free radical, hepatic glucose output, glucose 6-phosphatase

The inhibition of hepatic glucose output (HGO) by hyperglycaemia is a well-known phenomenon [1]. Recently it has been noticed that this phenomenon is caused by glucose-mediated autoregulation rather than by an effect of insulin. The direct glucose 6-

phosphatase (G6Pase) inhibitory effect of hyperglycaemia has also been demonstrated by using the hyperglycaemic clamp method [2]. The inhibition of G6Pase might explain the autoregulation of HGO since the key enzyme of glucose output is G6Pase whose activity enables the release of glucose from the hepatic cells. A possible means of inhibition of G6Pase induced by hyperglycaemia is by inhibition by a metabolic intermediate. However, the investigation of as many as 25 glucose metabolites did not reveal one that could have inhibited the G6Pase at physiological or pathophysiological concentrations [3]. The activity of the enzyme can also be inhibited by lipid peroxidation [4]. There is evidence indicating that hyperglycaemia alters the intracellular redox status. The NADH/NAD⁺ ratio increases, a phenomenon

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Abbreviations: BHT, Butylated hydroxytoluene; DMSO, dimethyl sulphoxide; G6Pase, glucose 6-phosphatase; GSH, glutathione (reduced form); HGO, hepatic glucose output; Pi, inorganic phosphate; SOD, superoxide dismutase.

referred to as hyperglycaemic pseudohypoxia because the same redox alteration can also be observed in hypoxic cells. Both hypoxia and pseudohypoxia can also be characterised by inducing free radical processes [5]. The increase in NADH/NAD⁺ ratio can also be observed in the cytoplasm of hepatic cells of hyperglycaemic rats (blood glucose level 30 mmol/l) [6].

The probability of the development of disorders associated with free radicals is also supported by the fact that parallel to the accumulation of the electron donor NADH in diabetes, disorders of iron metabolism can also be present [7]. Iron, which is capable of receiving and releasing electrons, may trigger lipid peroxidation and superoxide free radical production in this redox cycle [4]. Based on the above, it was hypothesized that by increasing the NADH concentration, elevated formation of free radicals would also occur and through free radical processes, hyperglycaemia may inhibit the activity of G6Pase and hence HGO. The present study was undertaken to provide evidence for this possibility:

Hyperglycaemia → NADH↑ → free radicals↑ → G6Pase activity↓ → HGO↓

An in vitro model was developed in which G6Pase activity of microsome fractions isolated from rat liver was assayed both in the presence and absence of NADH and iron complexes. For the identification of the nature of free radicals produced, SOD (a dismutating enzyme for superoxide free radicals), catalase (a decomposing enzyme of hydrogen peroxide), desferrioxamine (an irreversible iron chelator), BHT and Trolox (inhibitors of lipid peroxidation), ethanol and dimethylsulphoxide (DMSO) (scavengers of hydroxyl free radical) as well as GSH (a non-specific scavenger) were administered.

Materials and methods

All reagents were of the highest grade available. The final concentrations used were 10 mmol/l G6Pase, 2600 U/ml catalase, 49 U/l SOD, 2 mmol/l desferrioxamine, 1.4 mmol/l NADH, 50 mmol/l DMSO, 100 mmol/l ethanol, 2 mmol/l GSH, 0.1 mmol/l BHT, 0.1 mmol/l Trolox.

Liver microsomes were isolated from male Sprague-Dawley rats weighing about 200 to 250 g. The livers were initially perfused with 0.9 % NaCl and rapidly removed and routinely homogenized in a solution containing 0.25 mol/l sucrose, 0.01 mol/l Tris-HCl (pH 7.4), 0.001 mol/l EDTA, 50 µmol/l desferrioxamine. The microsomes were isolated by differential centrifugation, washed twice with 0.125 mol/l KCl in the absence of EDTA at 100 000 g, and resuspended in 0.125 mol/l KCl and stored at -75 °C.

The ferric complexes were prepared by dissolving ferric ammonium sulfate in 0.1 mol/l HCl, and then diluting with the respective chelator to the appropriate stock concentration. Ferric-ATP was utilized as a 1:20 complex, whereas ferric-EDTA was utilized as a 1:2 complex.

Isolated microsomes were incubated with or without 50 mmol/l Tris-HCl (pH 7.4) buffer with 10 mmol/l G6Pase in the presence or absence of 1.4 mmol/l NADH, 100 µmol/l iron complexes and scavengers over a 10–60 min time period at 37 °C. Control incubations with all of the reagents but without microsomes showed no NADH oxidation measuring at 340 nm (the concentration of the remaining NADH, in four independent measurements in ferric-ATP + Tris-HCl medium after 60 min was 98.8 ± 4.0 % (mean ± SD), in Tris-HCl, without ferric-ATP 102.1 ± 4.8 %, and in ferric-EDTA, without Tris-HCl at pH 7.4, 98.7 ± 2.2 %). The reaction was stopped by addition of 0.5 ml of trichloroacetic acid (10 % final concentration). The samples were centrifuged at 4 °C for 14 min. We used 1 ml of the supernatant for the determination of inorganic phosphate using the assay described by LeBel et al. [8]. Protein was determined by the method of Lowry et al. [9].

Statistical analysis. All results are from three experiments carried out in duplicate. Values refer to mean ± SD. Statistical analysis was carried out using one way repeated measures ANOVA of the SigmaStat for Windows, Version 1.0.

Results

Figure 1 illustrates Pi release as a measure of G6Pase enzyme activity with respect to time. By the end of the incubation period the enzyme activity was significantly reduced by the presence of NADH (30 min, $p < 0.03$; 40 min, $p < 0.02$, 50 min, $p < 0.01$, control vs NADH). Pi release was also significantly reduced by ferric-ATP + NADH (30, 40, 50 min $p < 0.01$, ferric-ATP vs ferric-ATP + NADH). Ferric-ATP + NADH, however, did not significantly lower G6Pase activity more than NADH did in the absence of ferric-ATP (at each stage $p > 0.05$).

When the incubation was carried out in the presence of ferric-EDTA, no inhibitory effect by NADH was observed. When Tris buffer, which is thought to have scavenger activity, was not used especially with respect to hydroxyl free radical, and pH was set at 7.4 by NaOH, a 36 % inhibition by NADH could be observed during min 60. (0.178 ± 0.012 vs 0.131 ± 0.04 µmol · l⁻¹ · mg protein⁻¹ · min⁻¹ Pi, ferric-EDTA vs ferric-EDTA + NADH, $p < 0.01$, data not shown).

Fig. 1. Inhibition of the activity of microsomal G6Pase by NADH and by ferric-ATP + NADH. Pi, Inorganic phosphate released by the enzyme from G6Pase, ▲ control, ● ferric-ATP, ◆ NADH, ■ ferric-ATP + NADH. (30 min, $p < 0.03$, 40 min, $p < 0.02$, 50 min, $p < 0.01$; control vs NADH and 30, 40, 50 min, $p < 0.01$; ferric-ATP vs ferric-ATP + NADH)

Fig. 2. Effect of scavengers on the inhibition of G6Pase enzyme evoked by NADH. Pi, Inorganic phosphate released by the enzyme from G6Pase; SOD, superoxide dismutase; DMSO, dimethyl sulphoxide; Desferal, desferrioxamine; BHT, butylated hydroxytoluene; GSH, reduced glutathione. ($p < 0.01$ control vs NADH, NADH vs NADH + Desferal, NADH + BHT, NADH + Trolox, NADH + GSH)

In order to analyse the potential free radical processes underlying G6Pase inhibition caused by NADH, scavengers were added to the Tris buffer incubation medium. The results are demonstrated in Figure 2. NADH inhibition was not influenced by SOD, catalase or DMSO.

However, the inhibitory effect of NADH was completely prevented by desferrioxamine, BHT, Trolox and GSH.

When NADH inhibition was studied in the presence of ferric-EDTA, SOD and BHT proved to be ineffective in preventing loss of G6Pase activity (0.131 ± 0.004 vs 0.130 ± 0.004 $\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ Pi, NADH vs NADH + SOD, 0.131 ± 0.004 vs 0.139 ± 0.011 $\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ Pi, NADH vs NADH + BHT, $p > 0.05$). However, radical scavengers, such as catalase (0.131 ± 0.004 vs 0.169 ± 0.014 $\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ Pi, NADH vs NADH + catalase, $p < 0.01$), DMSO (0.131 ± 0.004 vs 0.152 ± 0.001 $\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ Pi, NADH vs NADH + DMSO, $p < 0.01$), ethanol (0.131 ± 0.004 vs 0.164 ± 0.003 $\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ Pi, NADH vs NADH + ethanol, $p < 0.01$), and GSH (0.131 ± 0.004 vs 0.178 ± 0.016 $\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ Pi, NADH vs NADH + GSH, $p < 0.01$) were found to have a protective effect against the decrease in enzyme activity in ferric-EDTA medium (data not shown).

Discussion

Our results suggest that under conditions imitating hyperglycaemia (an increase in NADH) the activity of G6Pase decreases. The degree of inhibition (26–33 %) shows a quantitative equivalence with the data obtained using in vivo hyperglycaemic clamp methodology (26 %) [2]. Using exogenous ferric-ATP, no significant increase in the inhibitory effect by

NADH was observed. This indicates that for the induction of the inhibitory effect, the presence of iron in trace amounts, mobilized by NADH from the microsomes containing ferritin [4], and partially originating from the buffers used in these experiments, is sufficient. The intracellular presence of this trace amount of mobilized iron is an essential component of our hypothesis, which may model the hyperglycaemia-induced pseudohypoxic processes. Small amounts of iron are known to remain in the microsome even after preparation in the presence of desferrioxamine [4]. The inhibition of G6Pase in the NADH medium is probably induced by lipid peroxidation since SOD, catalase and DMSO (which remove superoxide, hydrogen peroxide and hydroxyl free radical) proved to be ineffective, while BHT, Trolox and GSH were found to be effective in protecting against inhibition.

When G6Pase activity was studied in the presence of ferric-EDTA complex (a powerful microsomal hydroxyl free radical generating system), no changes were found in the presence of NADH, due to the hydroxyl free radical scavenger properties of Tris-HCL buffer. In the absence of Tris buffer a decrease in the enzyme activity was observed in the presence of NADH, which was probably due to hydrogen peroxide and/or hydroxyl free radicals, since the decrease was catalase-sensitive, DMSO and ethanol reduced the damage to G6Pase, whereas SOD and BHT were found to be ineffective.

The three inhibitory factors of HGO are hyperglycaemia, insulin and parasympathetic stimulation [1]. The latter reflects acetylcholine signal transduction, the second messenger of which is nitric oxide. Nitric oxide may also play a role in the development of insulin action [10]. Nitric oxide is capable of binding with superoxide free radical with high affinity. It can be assumed that in our experiments hydrogen peroxide was produced through the dismutation of superoxide free radicals in the ferric-EDTA medium. As a result of the nitric oxide plus superoxide reaction peroxynitrite is produced, which, unlike nitric oxide, is incapable of activating guanylate cyclase and thus the agonist effect does not develop. Thus, parasympathetic and insulin-mediated HGO inhibition may be influenced by previous hyperglycaemic conditions which by inducing the production of free radicals, might cause disinhibition. As a result of these processes, normal, decreased or increased HGO levels can be detected in diabetic patients at any given moment. To test this in vivo, we plan to perform experiments using nitric oxide synthase substrate L-arginine and free radical scavengers in human.

We conclude that the decrease of HGO induced by hyperglycaemia can be caused by lipid peroxidation and/or by hydroxyl free radical due to an increase in NADH resulting in G6Pase inhibition.

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