Nitric oxide inhibits glycogen synthesis in isolated rat hepatocytes

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There is increasing evidence for the existence of intrahepatic regulation of glucose metabolism by Kupffer cell products. Nitric oxide (NO) is known to inhibit gluconeogenic flux through pyruvate carboxylase and phosphoenolpyruvate carboxykinase. However, NO may also influence glucose metabolism at other levels. Using hepatocytes from fasted rats incubated with the NO-donor *S*-nitroso-*N*-acetylpenicillamine, we have now found that the synthesis of glycogen from glucose is even more sensitive to inhibition by NO than gluconeogenesis. Inhibition of glycogen production by NO was accompanied by a rise in intracellular glucose 6-phosphate and UDPglucose. Activity of glycogen synthase, as measured in extracts of hepatocytes after the cells had been exposed to NO, was decreased. Experiments with gel-filtered liver extracts revealed that inhibition of glycogen synthase was caused by an inhibitory effect of NO on the conversion of glycogen synthase *b* into glycogen synthase *a*.

INTRODUCTION

The NO synthases catalyse the conversion of L-arginine into L-citrulline and nitric oxide (NO). NO is a short-lived metabolite with multiple biological activities [1]. Among the many biological effects of NO is a modulation of multiple pathways in glucose metabolism. In macrophages, NO synthase activity is associated with higher rates of glucose uptake, and metabolism through glycolysis and in the suppression of tricarboxylic acid cycle activity [2]. In isolated rat skeletal muscle NO may be a potential mediator of exercise-induced glucose transport [3,4] and is an inhibitor of insulin-stimulated, but not of basal, glycogen synthesis [4]. In rat hepatocytes incubated in the presence of NO donors a time- and dose-dependent inhibition of glucose synthesis from lactate and pyruvate has been reported which has been ascribed to an inhibition of phosphoenolpyruvate carboxykinase [5–8]. Nitric oxide also seems to modulate glycogenolysis. In perfused livers from fed rats, either no effect [9], inhibition [9] or stimulation [10] of glycogenolysis have been observed, depending on the experimental conditions used. In hepatocytes isolated from fed rats, NO slightly inhibited glucagon-stimulated, but not basal, glycogenolysis [11]. So far, no studies on the effect of NO on hepatic glycogen synthesis have appeared.

Using hepatocytes isolated from fasted rats, in which glycogen synthesis was stimulated by amino-acid-induced cell swelling [12,13], we have studied the mechanism of action of NO on glycogen synthesis. Since at high concentration NO is known to interfere with mitochondrial ATP production [14], and thus with ATP-dependent processes, experiments were performed under conditions where intracellular ATP was not modified. The data show that NO strongly inhibits glycogen synthesis and that this is owing to an inhibition of the conversion of glycogen synthase *b* into glycogen synthase *a*.

MATERIALS AND METHODS

Materials

All chemicals and enzymes were from either Boehringer (Mannheim, Germany) or Sigma (St. Louis, MO, U.S.A.). Stock solutions of *S*-nitroso-*N*-acetylpenicillamine (SNAP) were prepared in Krebs–Henseleit bicarbonate buffer and were immediately used.

Male Wistar rats (200–250 g) were obtained from T. N. O., Zeist, The Netherlands, and were maintained on standard laboratory chow and water *ad libitum*, until initiation of the fasting period.

Preparation of hepatocytes

Hepatocytes were isolated from animals fasted for 16–20 h, by collagenase perfusion, as described by Groen et al. [15].

Hepatocyte incubation

Hepatocytes (5–10 mg dry mass/ml) were incubated in Krebs–Henseleit hydrogencarbonate buffer fortified with 10 mM Na Hepes (pH 7.4), and the components indicated in the legends to Figures and Tables; final volume, 2–4 ml. The atmosphere was O_2/CO_2 (19:1, v/v); temperature, 37 °C. The structural integrity of the cells was checked before and after incubation by exclusion of Trypan Blue (0.25 %, w/v), which always exceeded 90 %.

When hepatocytes were incubated in a hypo-osmotic medium, the concentration of NaCl in the Krebs–Henseleit bicarbonate medium was decreased from 120 to 70 mM [12]. To check that the inhibitory effects of SNAP on metabolism were owing to NO and not to the presence of the other part of the molecule, control experiments were carried out in the presence of the parent compound *N*-acetylpenicillamine. In none of the experiments described did *N*-acetylpenicillamine have any effect (data not shown).

At the end of the incubations, samples of the suspension were taken and prepared for metabolite analysis. For the determination of glucose, 0.5 ml samples were quenched with $HClO_4$ (final concentration: 3.5 % w/v). The precipitated protein was removed by rapid centrifugation in the cold in a microcentrifuge, and the supernatants were neutralized to pH 7 by addition of a mixture of 2 M KOH plus 0.3 M Mops.

For determination of intracellular ATP, glucose 6-phosphate, UDPglucose and glutamate, cells (0.7 ml of the cell suspension)

Abbreviation used: SNAP, *S*-nitroso-*N*-acetylpenicillamine.

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were subjected to centrifugation through a layer of silicone oil (0.7 ml AR 200, Wacker Chemie) into a layer (0.25 ml) of $HClO_4$ (15 %, w/v). The acid cell extracts were neutralized with 2 M KOH and 0.3 M Mops to pH 7.

For the determination of glycogen, 0.3 ml of cell suspension was added to 1.5 ml ice-cold 150 mM NaCl plus 10 mM Na Hepes (pH 7.4) and the cells were centrifuged for 1 s in a microcentrifuge. After removal of the extracellular fluid, the cell pellet was extracted with 0.3 ml of 0.1 M KOH, incubated for 30 min at 90 °C, cooled and brought to pH 4.7 by addition of 3 M acetic acid. Precipitated protein was removed by centrifugation for 1 min in a microcentrifuge.

Samples for the determination of glycogen synthase and phosphorylase were prepared as follows: 0.3 ml of the cell suspension was added to 1.5 ml ice-cold 150 mM NaCl plus 10 mM Na Hepes (pH 7.4) and the cells were centrifuged for 1 s in a microcentrifuge. After removal of the supernatant, cells were extracted with 0.3 ml of a medium containing 50 mM glycylglycine, pH 7.4, 75 mM NaF, 3 mM EDTA, 0.5 % glycogen and 0.1 % Triton X-100, and were immediately frozen in liquid nitrogen [16].

Cell volume was determined with the 'hepatocrit' method [17].

Metabolite assays

Glucose was measured spectrophotometrically with ATP, $NADP^+$, glucose 6-phosphate dehydrogenase and hexokinase [18]. UDPglucose was determined fluorimetrically with NAD^+ and UDPglucose dehydrogenase [18]. ATP was measured fluorimetrically using glucose, $NADP^+$, glucose 6-phosphate dehydrogenase and hexokinase [19]. Glucose 6-phosphate was determined fluorimetrically with $NADP^+$ and glucose 6-phosphate dehydrogenase [19]. Glycogen was determined as glucose by treating the samples with amyloglucosidase at pH 4.7 [20]. The glucose produced was then measured at pH 7.4 as described above, except that the measurement was carried out fluorimetrically. Glutamate was measured with a Pharmacia-LKB alpha plus amino acid analyser using a lithium citrate buffer system.

Enzyme assays

Glycogen synthases *a* and *a*+*b* were measured with UDP[^{14}C]glucose and glycogen, as described by Lavoinne et al. [20]. Glycogen phosphorylase was measured with [^{14}C]glucose 1-phosphate and glycogen, as described by Hue et al. [16]. Total phosphorylase (*a*+*b*) was measured in the presence of 1,2-dimethoxyethane [21]. Glycogen synthase phosphatase was determined at 25 °C in a Sephadex G-25-filtered liver extract by following the activation of glycogen synthase as a function of time, as described previously except that the dithiothreitol concentration was 0.05 instead of 0.5 mM [13]. The livers were obtained from fed rats that had been treated with glucagon 10 min prior to removal of the livers, to increase the amount of inactive, phosphorylated glycogen synthase [13].

Statistical analysis

Data are summarized as means ± S.E. The statistical significance of differences of the means was calculated using Student's *t* test for paired groups of data.

RESULTS

Before studying the interaction between the NO donor SNAP and glycogen synthesis we wanted to obtain information on the ability of SNAP to inhibit gluconeogenesis under our experimental conditions and to compare these data with literature values. As shown in Table 1, 0.25 and 0.5 mM SNAP decreased glucose production from lactate by 28 % and 56 %, respectively. The same concentrations of SNAP hardly affected gluconeogenesis from dihydroxyacetone (Table 1). In these experiments, the level of intracellular ATP was not significantly affected by the concentrations of SNAP used (not shown). These results are in agreement with those published previously by Horton et al. [5].

Table 1 Effect of SNAP on gluconeogenesis

Hepatocytes were incubated for 1 h with either 10 mM lactate or 10 mM dihydroxyacetone. Data are the means (± S.E.) with the number of different hepatocyte preparations in parentheses.

	Glucose production (μmol/g dry mass per h)	
SNAP (mM)	Lactate	Dihydroxyacetone
0	166.7 ± 20.5 (4)	277.2 ± 16.8 (7)
0.25	119.6 ± 18.3* (4)	262.2 ± 13.9 (7)
0.5	73.9 ± 15.0* (4)	241.0 ± 15.4* (7)

* Significantly different from the control in the absence of SNAP ($p < 0.05$).

We next studied the effect of SNAP on glycogen synthesis. Because, in isolated rat hepatocytes, synthesis of glycogen from lactate or dihydroxyacetone alone is very low (data not shown, but see [12] and [22]) this process was studied in the presence of dihydroxyacetone in combination with either hypo-osmotic or amino-acid-induced cell swelling. An increase in hepatocyte volume is known to stimulate glycogen synthase [12] by activation of glycogen synthase phosphatase [13]. Amino-acid-induced cell swelling was brought about by addition of either 10 mM glutamine or 10 mM proline. As shown in Table 2, under all conditions tested, SNAP strongly inhibited production of glycogen, with almost complete inhibition being obtained at a concentration of 0.5 mM.

Because glucose formation from dihydroxyacetone was hardly affected by SNAP (Table 1), inhibition of glycogen formation must have been at a level beyond the production of glucose 6-phosphate. This was confirmed in another set of experiments in which dihydroxyacetone was replaced by glucose as a precursor for glycogen synthesis. Also in this case, glycogen production was almost completely inhibited by 0.5 mM SNAP (Table 2).

To obtain further information on the point of interaction of SNAP with the glycogen synthesizing pathway, the following protocol was developed. Hepatocytes were preincubated for 30 min in the presence of glucose and proline. This was followed by addition of 0.5 mM SNAP. Samples were then withdrawn every 10 min to up to 70 min of incubation and several metabolic parameters were analysed. Addition of SNAP completely inhibited synthesis of glycogen with a lag period of about 20 min (Figure 1A). Intracellular ATP levels were not affected (Figure 1B). Both intracellular levels of glucose 6-phosphate and UDPglucose increased upon addition of SNAP (Figure 1C and 1D). Activity of glycogen synthase *a* was relatively high in the presence of proline (cf. [12]) and both glycogen synthase *a* and glycogen synthase *a*+*b* activity immediately declined after SNAP addition (Figure 1E and 1F). By contrast, phosphorylase *a* activity was relatively low (cf. ref. 12) and neither phosphorylase *a* nor phosphorylase *a*+*b* activities were significantly affected by

Table 2 Inhibition of glycogen synthesis by SNAP

Hepatocytes were incubated for 1 h in the presence of either 10 mM dihydroxyacetone or 20 mM glucose, and the concentrations of SNAP indicated. To stimulate glycogen synthesis from these substrates, 10 mM glutamine or 10 mM proline was also added, or hepatocytes were incubated under hypo-osmotic conditions. Data are the means (±S.E.) with the number of different hepatocyte preparations in parentheses.

Additions	SNAP (mM)	Glycogen synthesis (μmol/g dry mass/h)		
		Glutamine	Proline	Hypo-osmotic
Dihydroxyacetone	0	59.4±3.2 (3)	62.9±0.5 (3)	30.0±3.5 (3)
	0.25	12.1±6.7* (3)	8.9±8.9* (3)	3.9±1.6* (3)
	0.5	3.0±1.4* (3)	3.4±0.7* (3)	0.7±0.6* (3)
Glucose	0	94.6±5.5 (5)	100.5±5.2 (6)	72.7±5.1 (4)
	0.25	38.9±16.5* (4)	42.4±15.2* (4)	29.0±8.9* (4)
	0.5	12.3±4.6* (5)	9.9±3.3* (6)	11.9±4.4* (4)

* Significantly different from the control in the absence of SNAP ($p < 0.05$).

SNAP (Figure 1G and 1H), with the exception of the last time point at which a significant increase in phosphorylase *a* activity was noted (Figure 1G).

Previously we have shown that, in addition to cell swelling, glutamate, a major catabolite of proline in hepatocytes [23], activates glycogen synthase phosphatase [13]. To rule out the possibility that SNAP interfered with the production of glutamate from proline oxidation, the effect of SNAP on the intracellular accumulation of glutamate was tested. The level of this amino acid was not affected by SNAP, nor was proline-induced cell swelling (data not shown).

Subsequently, the effect of SNAP on the activity of glycogen synthase phosphatase and on glycogen synthase was measured in a cell extract. For this purpose we used an extract of a liver obtained from a fed rat that had received glucagon intravenously 10 min prior to removal of the liver [13]. The extract was filtered through Sephadex G25 to remove small molecular mass components. In this preparation, glycogen synthase is mainly in the phosphorylated, inactive, *b* form, and the activity of glycogen synthase phosphatase can be followed as the appearance of (active) glycogen synthase *a* as a function of time [13,24]. The activity of glycogen synthase phosphatase was tested in the absence and in the presence of 3 mM AMP and 5 mM $MgCl_2$. These compounds relieve the inhibition of glycogen synthase phosphatase by phosphorylase *a* [24]. The activity of glycogen synthase phosphatase was indeed higher in the presence of AMP and Mg^{2+} than in their absence (cf. Figure 2A and 2B). Addition

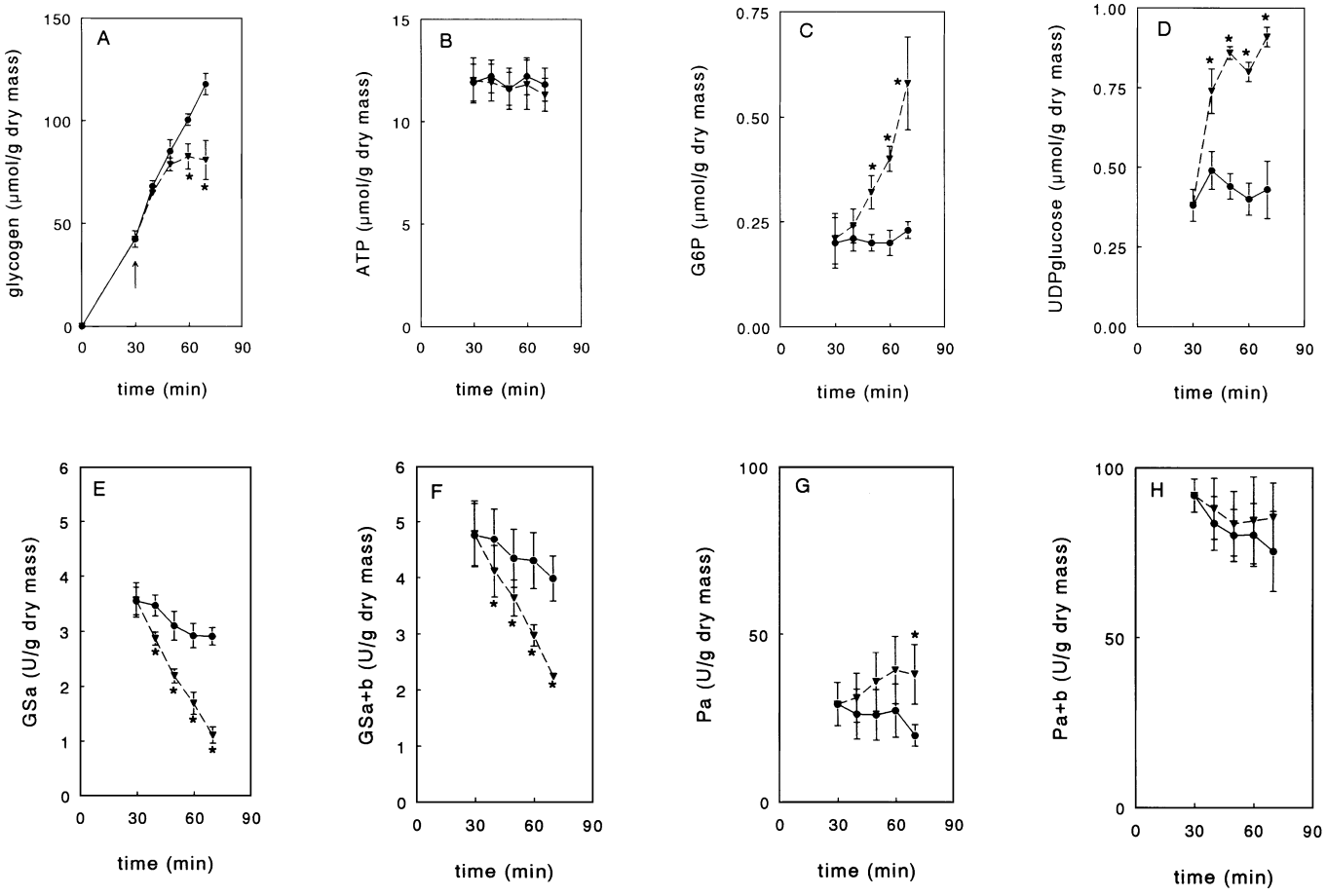

Figure 1 Interaction of SNAP with the glycogen synthesizing pathway

The effect of SNAP is shown on (**A**) glycogen synthesis, (**B**) ATP levels, the intracellular levels of (**C**) glucose 6-phosphate and (**D**) UDPglucose, and on the activity of (**E**) glycogen synthase *a*, (**F**) glycogen synthase *a*+*b*, (**G**) phosporylase *a* and (**H**) phosporylase *a*+*b*. Hepatocytes were incubated with 20 mM glucose and 10 mM proline. After 30 min (arrow), 0.5 mM SNAP was added and incubation was continued. Samples were withdrawn every 10 min, as indicated, and analysed for metabolites and enzymes activities. (●) Control; (▼) SNAP present. Data are the means (±S.E.) of experiments carried out with four different hepatocyte preparations. *Significantly different from the control in the absence of SNAP ($p < 0.05$).

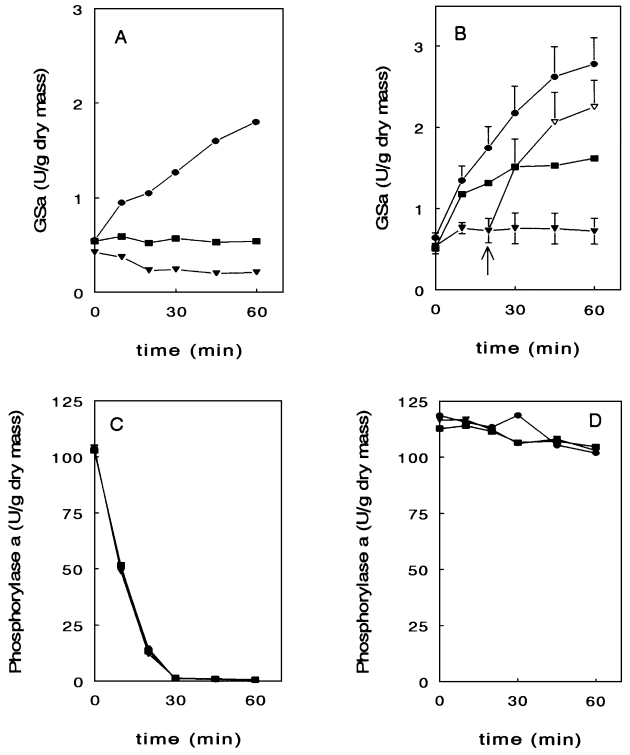

Figure 2 Inhibition of glycogen synthase phosphatase by SNAP

A gel-filtered liver extract was incubated, in the absence (●) or presence of 0.25 mM (■) or 0.5 mM (▼) SNAP. At the times indicated, samples were withdrawn and assayed for glycogen synthase (GS) *a* (**A**,**B**) and phosphorylase *a* (**C**,**D**) activity. In **B** and **D**, 3 mM AMP and 5 mM $MgCl_2$ were also present. In **B**, 5 mM reduced glutathione was added (arrow) 20 min after the liver extract had been incubated in the presence of 0.5 mM SNAP (▽). Data are the means of experiments carried out with two (**A**, **C** and **D**) or three (**B**) different liver extracts. Error bars are only indicated for experiments in which three liver extracts were used.

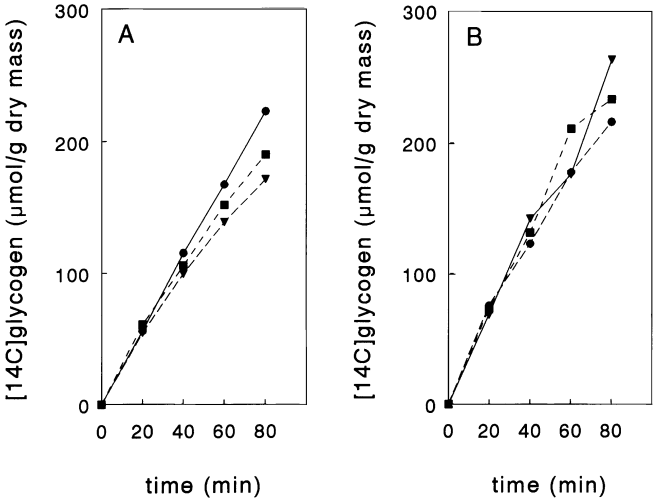

Figure 3 Effect of SNAP on glycogen synthase *a*

A gel-filtered liver extract was preincubated for 1 h to allow conversion of glycogen synthase *b* into *a* (in the absence of AMP and Mg^{2+}). After this period, glycogen synthase phosphatase was stopped by addition of 25 mM fluoride [13], and glycogen synthase *a* activity was measured with [^{14}C]UDPglucose and glycogen, in the absence (●) or presence of 0.25 mM (■) or 0.5 mM (▼) SNAP. (**A**) Synthesis of [^{14}C]glycogen was measured in the presence of 10 mM sulphate (glycogen synthase *a*); (**B**) synthesis of [^{14}C]glycogen was measured in the presence of 10 mM glucose 6-phosphate (glycogen synthase *a*+*b*). Data are the means of experiments carried out with two different liver extracts.

of 0.25 mM SNAP completely inhibited glycogen synthase phosphatase in the absence of AMP and Mg^{2+} (Figure 2A) while 0.5 mM SNAP was required for complete inhibition in the presence of AMP and Mg^{2+} (Figure 2B). By contrast, neither phosphorylase inactivation (in the absence of AMP and Mg^{2+}) (Figure 2C) nor phosphorylase activity in the presence of AMP and Mg^{2+} (Figure 2D) were affected by SNAP. SNAP had little effect on the low activity of glycogen synthase present at the start of the experiment (zero time, Figure 2A and 2B), indicating that SNAP had little effect on glycogen synthase *a* itself. This was also confirmed in the following manner. The liver extract was first preincubated for 60 min to allow conversion of glycogen synthase *b* into *a*. After this period, about 85% of the enzyme was in the active form (cf. Figure 3A and 3B). When, after this conversion, SNAP was added, only a slight inhibition of glycogen synthase activity was observed (Figure 3A and 3B).

These data led us to conclude that SNAP primarily inhibits glycogen synthase phosphatase, with little effect on the active form of glycogen synthase. The inhibition by SNAP of glycogen synthase phosphatase in gel-filtered liver extracts could be reversed by addition of reduced glutathione (Figure 2B) or dithiothreitol (not shown).

DISCUSSION

Our results confirm previous reports on a dose-dependent inhibition by NO of gluconeogenesis from lactate [5–8]. In agreement with the data of Titheradge et al. [7] there was little inhibition of gluconeogenesis from dihydroxyacetone by SNAP at the concentrations used. Concentrations of SNAP higher than 0.5 mM could not be used because these caused intracellular ATP to decrease (not shown).

The results of the present study provide the first evidence for a potential role of NO in the regulation of glycogen synthesis in hepatocytes. Addition of the NO donor SNAP to freshly isolated rat hepatocytes strongly inhibited glycogen synthesis. There is no doubt that this was caused by inhibition of flux through glycogen synthase. Not only was there a decrease in enzyme activity when measured in cell lysates after incubation of the cells with the NO donor, but simultaneously both intracellular glucose 6-phosphate and UDPglucose increased. The latter observation illustrates that the intact hepatocyte flux through glycogen synthase was, indeed, decreased by addition of SNAP.

The kinetics of inhibition of glycogen synthesis by SNAP deserve comment. Immediately after addition of SNAP, intracellular UDPglucose increased (Figure 1D), followed 10 min later by an increase in glucose 6-phosphate (Figure 1C). Synthesis of glycogen, however, did not cease until 20 min after SNAP addition (Figure 1A). Apparently, the increase of UDPglucose, substrate of glycogen synthase, and the increase of glucose 6-phosphate, activator of both glycogen synthase *b* and glycogen synthase phosphatase [25], initially compensated for the inhibition of glycogen synthase phosphatase by NO.

At the concentrations of SNAP used, hepatocyte phosphorylase activity was not significantly affected (Figure 1G), in agreement with similar data by Borgs et al. [10] who studied the effect of NO gas on glycogenolysis in hepatocytes from fed rats. The fact that in our experiments glycogen, already synthesized by the cells before SNAP addition, was not degraded but remained constant after SNAP addition (Figure 1A), also shows that

phosphorylase flux in the intact hepatocyte must have been low under these conditions. It must be stressed that this conclusion only holds for low concentrations of SNAP. When concentrations higher than 0.5 mM were used, phosphorylase became activated and glycogen already synthesized was degraded (data not shown). Because under these conditions intracellular ATP dropped, it is likely that phosphorylase increased because of an increase in cytosolic Ca^{2+}, which is known to activate phosphorylase kinase in hepatocytes [26,27].

In contrast to the situation in isolated hepatocytes, in livers from fed rats, perfused with a solution containing 1 mM glucose, Borgs et al. [10] observed a transient stimulation of glycogenolysis which was ascribed to a partial activation of glycogen phosphorylase. This NO effect in the perfused liver was blocked by co-administration of cyclooxygenase inhibitors, suggesting a role for prostanoids produced by the non-parenchymal cells in the glycogenolytic response of the parenchymal cells to NO [10].

Glycogen synthase *a* was only very slightly affected by SNAP (Figure 3A). The inhibition of glycogen synthase phosphatase by SNAP (Figure 2) can therefore be owing to either direct inhibition of this enzyme or, alternatively, it is caused by binding of NO to the inactive form of glycogen synthase, precluding its activation. A combination of both mechanisms, however, is also possible. Inhibition of glycogen synthase *b* would have two effects: firstly, less glycogen synthase *a* can be formed by dephosphorylation and, secondly, the activity of glycogen synthase *b* in the presence of glucose 6-phosphate will be less. Total glycogen synthase $a+b$ indeed declined in the presence of SNAP (Figure 1F). In this context it is of interest to note that oxidized glutathione has been reported to inactivate muscle glycogen synthase *b* by decreasing its affinity for glucose 6-phosphate, while reduced glutathione protects against this inactivation [28]. The fact that inhibition of glycogen synthase phosphatase by SNAP could be reversed by addition of reduced glutathione (Figure 2B) or of dithiothreitol would suggest that the same –SH group(s) in glycogen synthase *b* that is (are) modified by oxidized glutathione is (are) also modified by NO.

In conclusion, glycogen synthesis in hepatocytes is strongly inhibited by NO, because this compound inhibits the conversion of glycogen synthase *b* into *a*. This mechanism must be added to the indirect activation of phosphorylase by NO which is mediated by increased prostaglandin synthesis in non-parenchymal cells [10]. Thus, there is the intriguing possibility that *in vivo* NO may function as an autocrine factor in the liver to modulate glucose production directly via its inhibitory effect on glycogen synthesis and gluconeogenesis and indirectly via its effect on the secretion of other intrahepatic mediators that promote glycogen breakdown.

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