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The action of extracellular NAD^+ on Ca^{2+} efflux, hemodynamics and some metabolic parameters in the isolated perfused rat liver

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Abstract

The action of NAD^+ on hemodynamics and metabolism of the isolated perfused rat liver was investigated. Extracellular NAD^+ (20–100 μM) stimulated glycogen breakdown (glucose release) and inhibited oxygen uptake. Lactate production was predominantly increased, and pyruvate production was predominantly inhibited. NAD^+ also increased the portal perfusion pressure. All metabolic effects were strictly Ca^{2+}-dependent. The effects were absent when Ca^{2+} was excluded, and reintroduction of the cation restored the effects. In preloaded livers, NAD^+ accelerated $^{45}Ca^{2+}$ efflux. The action of NAD^+ was sensitive to three inhibitors of eicosanoid synthesis, suggesting that this action is mediated by these compounds, which are known to be produced and released by Kupffer and endothelial cells. It is impossible to infer from the available data if NAD^+ exerts all these effects by itself or if they are caused by one or more of its extracellular hydrolysis products. Nicotinamide was ineffective and can be excluded, but especially cyclic ADP-ribose and ADP-ribose are possibilities that should be considered in future work.

Keywords: NAD^+; Cyclic ADP-ribose; Liver perfusion; Hemodynamics; Metabolism

1. Introduction

The role of the nicotinamide adenine nucleotides (NAD^+ and $NADP^+$) in the transfer of reducing equivalents is textbook knowledge. In the last years, it became also evident that these molecules are involved in intracellular signalling by virtue of enzyme-catalyzed transformations (Lee, 1994; Ziegler, 2000). One of these transformations is ADP-ribosylation (ADP ribosyl-transferase), which consists of the direct transfer of the ADP-ribose moiety of $NAD(P)^+$ to an acceptor protein, with a consequent modification of its biological properties. Another important transformation is catalyzed by the NAD^+ glycohydrolases/ADP-ribosyl cyclases. When acting as a hydrolase, this enzymatic system generates ADP-ribose and free nicotinamide. However, it can also form cyclic ADP-ribose, a substance which has been recognized as Ca^{2+}-mobilizing agent in sea urchin eggs (Clapper et al., 1987) as well as in several mammalian cells (Lee, 1994; Ziegler, 2000; Koshiyama et al., 1991). NAD^+ glycohydrolases/ADP-ribosyl cyclases have been identified in a great number of organisms including prokaryotes and eukaryotes. Most ADP-ribosyl cyclases (also known as CD38 proteins) are transmembrane glycoproteins whose catalytic activity is located on the extracellular carboxyl domain, but several intracellular forms have also been characterized (Ziegler, 2000).

It is since long known that extracellular NAD^+ can be rapidly hydrolyzed to ADP-ribose in the isolated perfused rat liver (Liersch et al., 1971). More recently, it has been shown that rabbit and rat liver extracts are able to catalyze the formation of cyclic ADP-ribose (Rusinko and Lee, 1989; Chini et al., 1997), the hepatic ADP-ribose cyclase activity being similar to that of other tissues. The existence of the CD38 protein in the plasma membrane of hepatocytes has been demonstrated by Khoo and Chang (2000). In the liver cells, this protein shows a 10-fold higher specific activity in the sinusoidal membrane fraction than in the bile canalicular membrane fraction.

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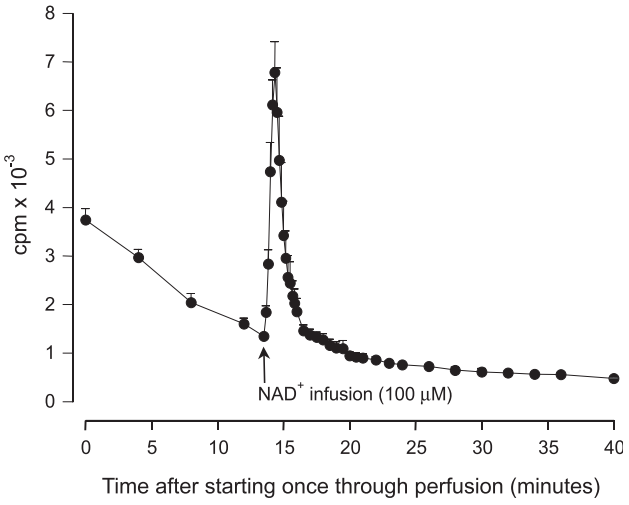

Fig. 1. $^{45}Ca^{2+}$ efflux induced by NAD^+. Livers from fed rats were perfused as described in Materials and methods. After labelling the intracellular calcium stores with $^{45}Ca^{2+}$ in the recirculating mode (see Materials and methods for details), perfusion was switched to the non-recirculating mode. NAD^+ was infused from 13.5 to 40 min perfusion time. Samples were taken for the measurement of $^{45}Ca^{2+}$ by means of liquid scintillation spectroscopy. Data are the means ± mean standard errors of three liver perfusion experiments.

Evidence seems to indicate, however, that hepatocytes do not possess cytosolic cyclic ADP-ribose sensitive Ca^{2+} stores. This has been shown in experiments with both permeabilized liver cells (Lilly and Gollan, 1995) and intact hepatocytes which were microinjected with cyclic ADP-ribose (Hirata et al., 2002). These experiments seem to suggest that the cytosol of hepatocytes does not respond directly to cyclic ADP-ribose, at least what Ca^{2+} movements concerns.

The use of isolated hepatocytes is evidently extremely useful for many purposes. However, the liver is not composed solely of hepatocytes, but by several other types of cells which are in either direct or indirect contact by means of the microcirculation. Interactions between cells have been demonstrated by several studies. For example, it is well known that endothelial and Kupffer cells are able to produce and release eicosanoids (Birmelin and Decker, 1984; Dieter et al., 1986) which are active on hemodynamics and also on metabolic fluxes in hepatocytes (Häussinger et al., 1988; Altin and Bygrave, 1988). Moreover, it has been reported that the NAD^+-glycohydrolase activity is 65-fold higher at the periphery of Kupffer cells than at the

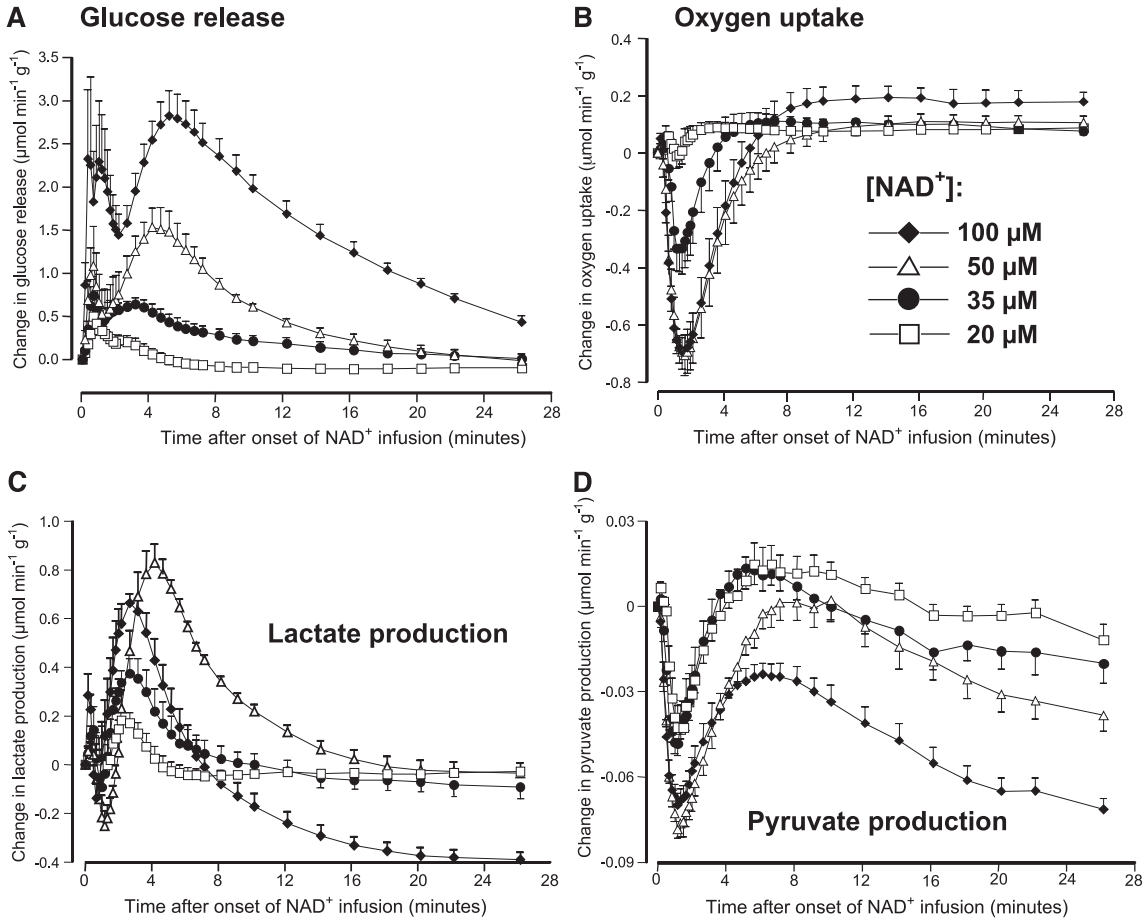

Fig. 2. Concentration dependence of the metabolic effects of NAD^+. Livers from fed rats were perfused as described in Materials and methods. After oxygen uptake stabilization, NAD^+ was infused during 26 min at four different concentrations, 20, 35, 50, and 100 µM, in separated experiments. Samples were taken for the measurement of glucose, lactate, and pyruvate. Oxygen was monitored polarographically. Data are represented as changes relative to the values just before NAD^+ infusion and are the means ± mean standard errors of four to eight liver perfusion experiments.

periphery of hepatocytes (Amar-Costesec et al., 1985). For these reasons, we decided to initiate a series of studies on the action of extracellularly infused NAD^+ in the perfused rat liver in which cell-to-cell interations and the microcirculation are both preserved. The rationale for using NAD^+ is based on the fact that ADP-ribosyl cyclase is an ectoenzyme, whose substrate is most probably extracellular NAD^+. In principle at least, the presence of extracellular NAD^+ may be required even though this may occur in a limited range of space and time. This can indeed be expected from the properties of the NAD^+ transport systems that have been described so far, whose activity is apparently under strong and complex control (Franco et al., 2001; Bruzzone et al., 2001).

The results that we have obtained revealed that extracellular NAD^+ is able to affect liver metabolism and hemodynamics in a Ca^{2+}-dependent manner, but that these effects are more likely the result of cell-to-cell interactions rather than an exclusive action on hepatocytes.

2. Materials and methods

2.1. Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. All enzymes and coenzymes used in the enzymatic assays, NAD^+, nordihydroguaiaretic acid, indomethacin, and bromophenacyl bromide were purchased from Sigma (St. Louis, USA). Radioactive calcium ($[^{45}Ca]CaCl_2$) was purchased from NEN Life Sciences Products (Boston, USA). All standard chemicals were from the best available grade (98–99.8% purity) and were purchased from Merck (Darmstadt, FRG), Carlo Erba (São Paulo, Brasil) and Reagen (Rio de Janeiro, Brazil).

2.2. Liver perfusion

Male Wistar rats, weighing 200–250 g, were fed ad libitum with a standard laboratory diet (Purina, São Paulo,

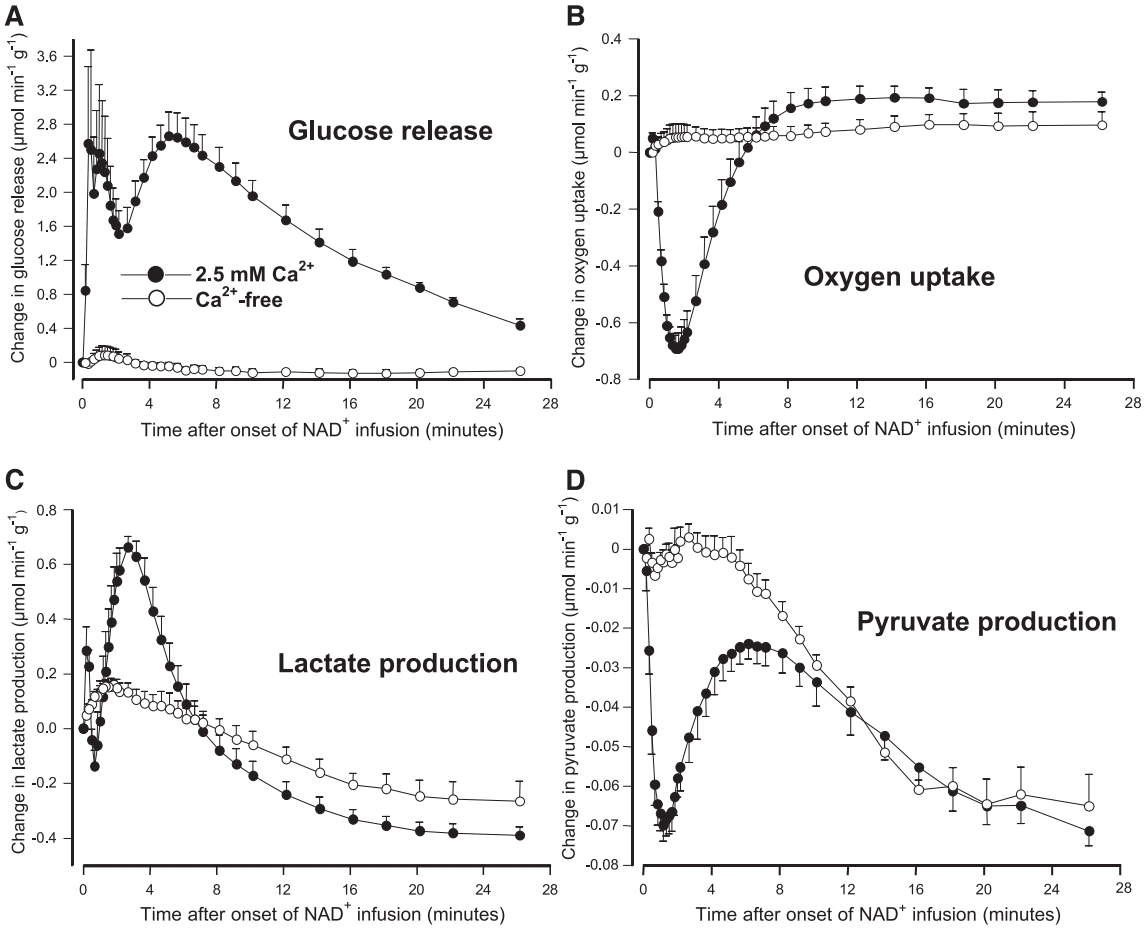

Fig. 3. Influence of Ca^{2+} on the metabolic effects of NAD^+. Livers from fed rats were perfused as described in Materials and methods. Ca^{2+} depletion was achieved by sucessive phenylephrine pulses, as described in Materials and methods. After depletion, perfusion was continued with Ca^{2+}-free Krebs/Henseleit-bicarbonate buffer. In the control experiments, Krebs/Henseileit-bicarbonate buffer containing 2.5 mM Ca^{2+} was used throughout. Samples were taken for the measurement of glucose, lactate, and pyruvate. Oxygen was monitored polarographically. Data are represented as changes relative to the values just before NAD^+ infusion and are the means ± mean standard errors of six to eight (control) or three (Ca^{2+}-free) liver perfusion experiments.

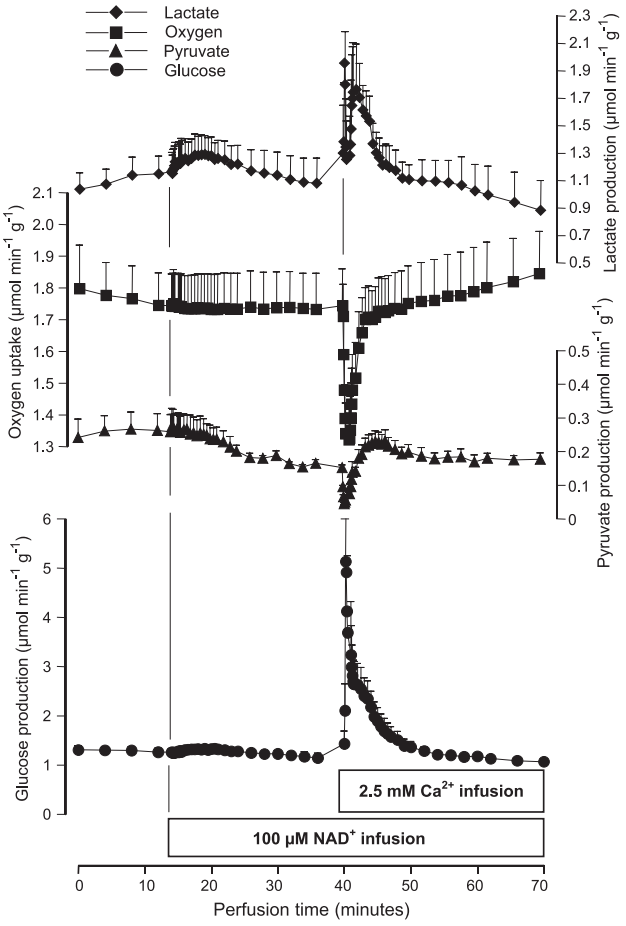

Fig. 4. Response of the liver to Ca^{2+} reintroduction in the presence of NAD^+. Livers from fed rats were perfused as described in Materials and methods. Ca^{2+} depletion was achieved by sucessive phenylephrine pulses, as described in Materials and methods. After the last phenylephrine pulse, perfusion was continued with Ca^{2+}-free Krebs/Henseleit-bicarbonate buffer, perfusate sampling was initiated (zero time), and 100 µM NAD^+ was infused (10 min) as indicated by the horizontal bar. At 40 min, 2.5 mM Ca^{2+} was introduced, as indicated by the horizontal bar. The data points are the means ± mean standard errors of three liver perfusion experiments.

Brazil). For the surgical procedure, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Hemoglobin-free, non-recirculating perfusion was done according to the technique described by Scholz and Bücher (1965).

After cannulation of the portal and cava veins, the liver was positioned in a Plexiglas chamber. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted between 30 and 35 ml min^{-1}, depending on the liver weight. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37 °C. The composition of the Krebs/Henseleit-bicarbonate buffer is the following: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4, and 2.5 mM $CaCl_2$.

For performing Ca^{2+}-free perfusion, the intracellular Ca^{2+} pools were exhausted. The following procedure was adopted. Livers were pre-perfused with Ca^{2+}-free Krebs/Henseleit-bicarbonate buffer containing 0.1 mM EGTA, 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate. In order to ensure maximal depletion of the intracellular Ca^{2+} pools, phenylephrine (2 µM) was infused repeatedly (four times) during short periods of 2 min, with intervals of 5 min. According to Reinhart et al. (1982), this procedure depletes the intracellular Ca^{2+}-pools which are normally mobilized when Ca^{2+}-agonists are infused.

For measuring Ca^{2+} efflux, the cellular stores were labelled with $^{45}Ca^{2+}$ (Yamamoto et al., 1992). At approximately 20 min after the surgical procedure, [^{45}Ca]$CaCl_2$ was added to 100 ml of the perfusion fluid (0.25 µCi/ml), and the system was switched to a recirculating one. Recirculation was continued for 40 min. After this time, the perfusion fluid was switched to Ca^{2+}-free Krebs/Henseleit-bicarbonate buffer containing 1 mM ethylene diamine tetraacetic acid (EDTA), and perfusion was continued in the open mode. Samples for the measurement of the outflowing radioactivity were collected, and NAD^+ was infused at the desired time interval.

2.3. Analytical

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents. The following compounds were assayed by means of standard enzymatic procedures: glucose (Bergmeyer and Bernt, 1974), lactate (Gutman and Wahlefeld, 1974), and pyruvate (Czok and Lamprecht, 1974). The oxygen concentration in the outflowing perfusate was monitored continuously, employing a Teflon-shielded platinum electrode adequately positioned in a Plexiglas chamber at the

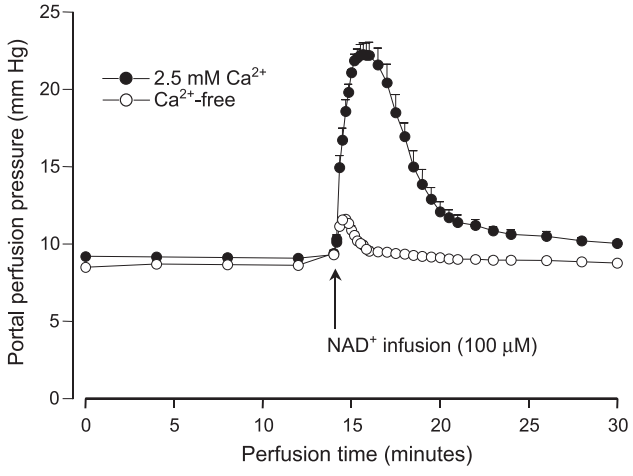

Fig. 5. Ca^{2+} dependence of the action of NAD^+ on portal perfusion pressure. Livers from fed rats were perfused as described in Materials and methods. NAD^+ was infused from 14 to 30 min. The perfusion pressure was monitored by means of a pressure transducer. Data are the means ± mean standard errors of three liver perfusion experiments.

exit of the perfusate (Bracht et al., 2003). Metabolic rates were calculated from input–output differences and the total flow rates and were referred to the wet weight of the liver.

The $^{45}Ca^{2+}$ radioactivity was measured by liquid scintillation spectroscopy. The following scintillation solution was used: toluene/Antarox (3:1) containing 5 g/l 2,5-diphenyloxazole and 0.1 g/l 2,2′ -*p*-phenylene-bis(5-phenyloxazole).

The portal perfusion pressure were monitored by means of a pressure transducer (Hugo Sachs Elektronic-Harvard Apparatus, March-Hugstetten, Germany). The sensor was positioned near the entry of the portal vein, and the transducer was connected to a recorder. The pressure changes were computed from the recorder tracings and expressed as millimeters Hg.

3. Results

In the first experiments, the capacity of NAD^+ to induce Ca^{2+} movements in the liver was investigated. The results are shown in Fig. 1. The intracellular Ca^{2+} stores of livers from fed rats were previously labelled with $^{45}Ca^{2+}$ as described in Materials and methods. Labelling was done by recirculating a $^{45}Ca^{2+}$-containing perfusion fluid during 40 min, and the time scale in Fig. 1 begins when the non-recirculating mode was restored. The introduction of 100 μM NAD^+ at 13.5 min produced a clear and pronounced transient increase in $^{45}Ca^{2+}$ efflux, the peak value exceeding the basal line by a factor of 5.0. The stimulation period did not exceed 3.5 min. This transient stimulation of Ca^{2+} release due to NAD^+ infusion was very similar to that one reported for other Ca^{2+}-mobilizing agonists (Yamamoto et al., 1992).

In the liver, agonists that are able to mobilize Ca^{2+} in the way illustrated by Fig. 1 generally are also able to affect several metabolic fluxes including glycogenolysis, glycolysis, and oxygen uptake (Altin and Bygrave, 1988; Yamamoto et al., 1992; Reinhart et al., 1984). In order to investigate this possibility for NAD^+, a series of experiments were carried out in which the compound was infused

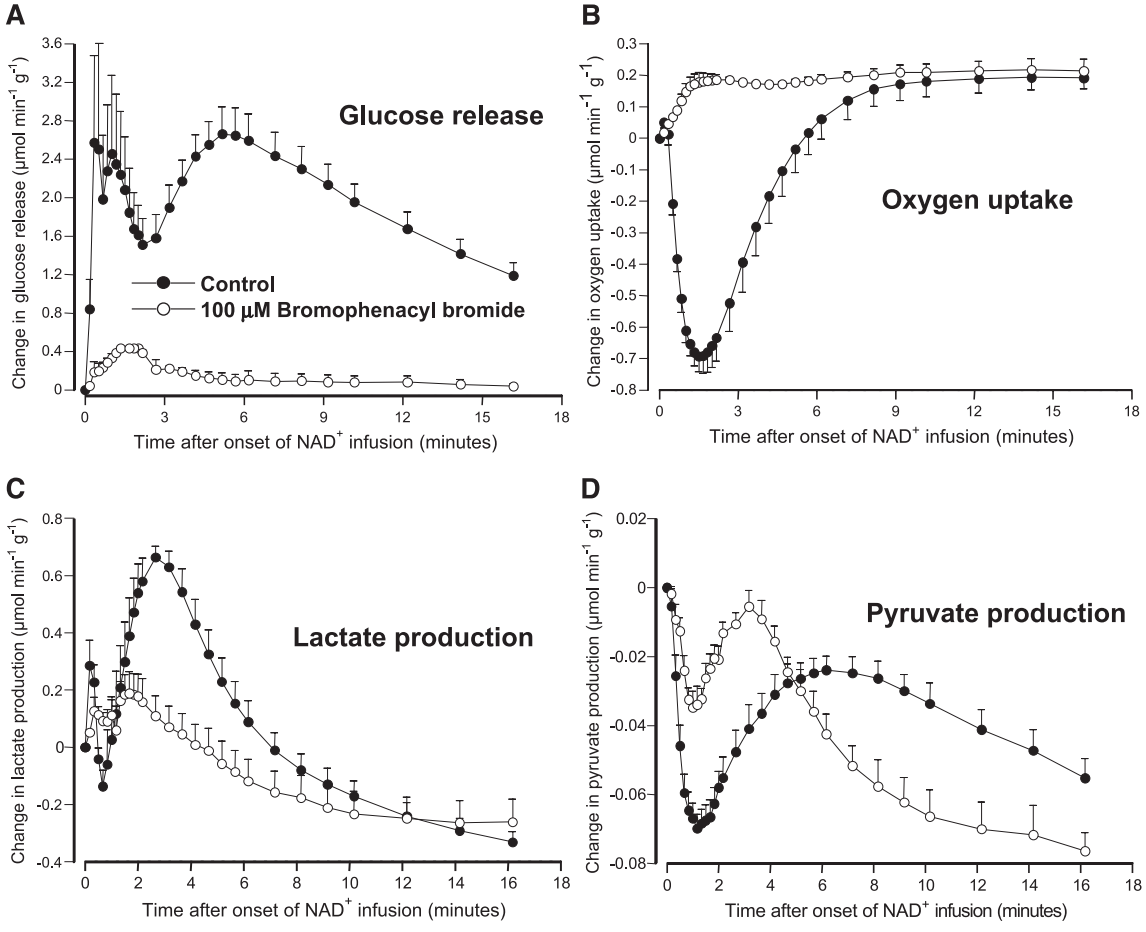

Fig. 6. Influence of bromophenacyl bromide on the metabolic effects of NAD^+. Livers from fed rats were perfused as described in Materials and methods. Bromophenacyl bromide was added to the Krebs/Henseleit bicarbonate buffer as a dimethylsulfoxide solution (250 μl/l) for a final concentration of 100 μM. Infusion of bromophenacyl bromide was started 16 min prior to the NAD^+ infusion (100 μM) and continued during the whole time of NAD^+ infusion. In the control experiments, Krebs/Henseleit-bicarbonate buffer containing 250 μl/l DMSO was infused during 16 min prior to the NAD^+ infusion. Samples were taken for the measurement of glucose, lactate, and pyruvate. Oxygen was monitored polarographically. Data are represented as changes relative to the values just before NAD^+ infusion and are the means ± mean standard errors of six to eight (control) or four (bromophenacyl bromide) liver perfusion experiments.

at various concentrations. Livers from fed rats were employed. These livers are releasing glucose at the expense of endogenous glycogen, degrading glucose 6-phosphate via the glycolytic pathway to lactate and pyruvate and respiring as a result of endogenous fatty acids oxidation (Scholz and Bücher, 1965). The results of these experiments are shown in Fig. 2, where the changes caused by NAD^+ were represented as a function of time after the onset of the infusion. Basal rates (metabolic rates before NAD^+ infusion) were subtracted. The mean basal rates of glucose release, oxygen consumption, lactate production, and pyruvate production were 1.05 ± 0.21, 1.83 ± 0.05, 0.89 ± 0.04, and 0.091 ± 0.007 μmol min^{-1} (g liver)$^{-1}$, respectively. The introduction of NAD^+ produced quite pronounced concentration-dependent changes, which presented complex kinetics. Glucose release was transiently increased by NAD^+ at the concentrations of 20, 35, 50, and 100 μM, with a biphasic kinetics. The first activation burst appeared immediately after starting infusion and reached its maximum at the end of the first minute, decaying rapidly thereafter. The second burst appeared more slowly, and its maximum occurred at different times, directly proportional to the NAD^+ concentrations. Its decay was also slow. The extent of the effects increased for each increase in concentration. In the passage from 50 to 100 μM, the effects almost doubled. Oxygen consumption was transiently inhibited by NAD^+. The extent of this effect increased for concentrations between 20 and 50 μM; from 50 to 100 μM, no increase was found. With 50 and 100 μM NAD^+, maximal inhibition occurred at 1.5 min after starting infusion. With lower concentrations, besides the smaller degree of inhibition, maximal inhibition tended to occur at earlier times after starting infusion. For all concentrations, inhibition had already vanished at 8 min infusion time. The effects of NAD^+ on lactate production presented a kinetics which was more complex than that one found for the effects on glucose release and oxygen uptake. An initial period of small fluctuations was followed by stimulation, which was transitory. The changes in lactate production increased with the NAD^+ concentration in the range up to 50 μM. With 100

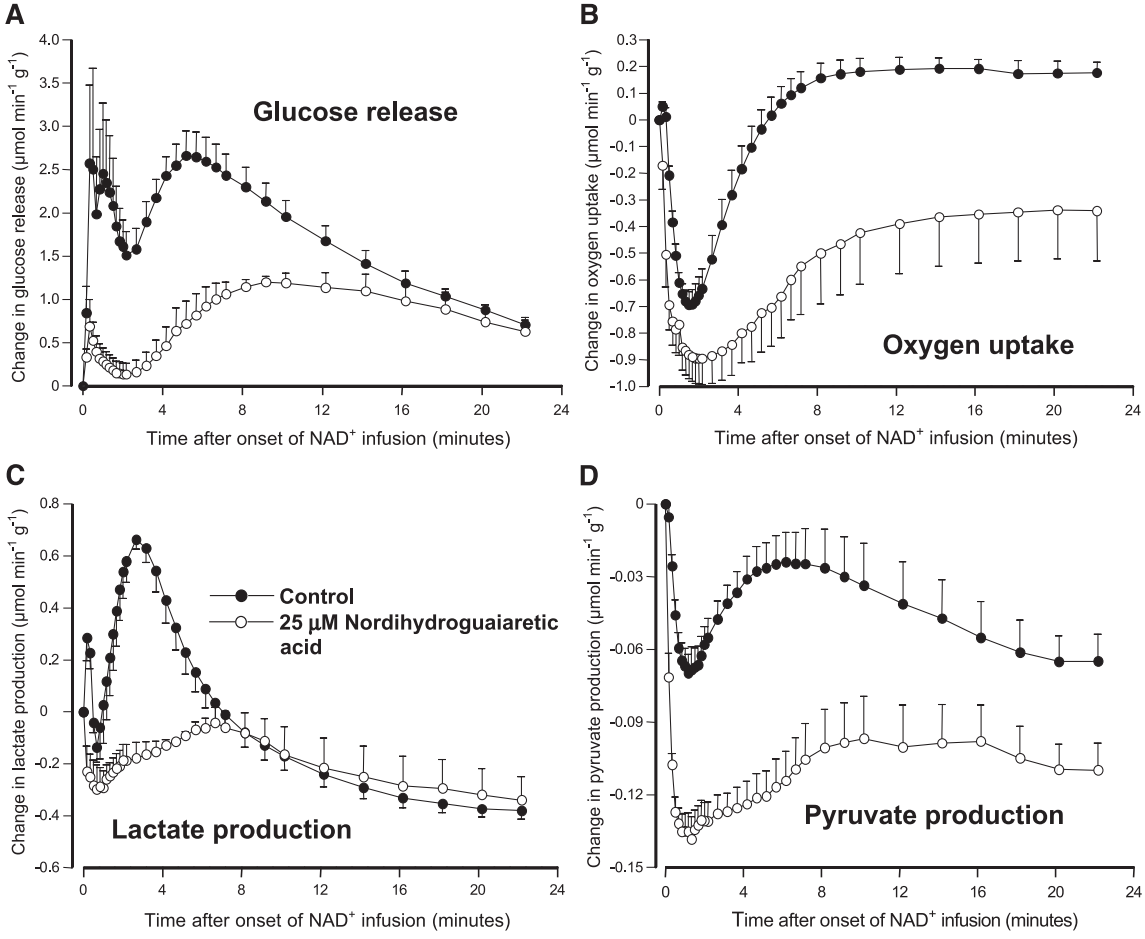

Fig. 7. Influence of nordihydroguaiaretic acid on the metabolic effects of NAD^+. Livers from fed rats were perfused as described in Materials and methods. The infusion of nordihydroguaiaretic acid (25 μM) was started 16 min prior to the NAD^+ infusion (100 μM) and continued until the end of the experiment. In the control experiments NAD^+ infusion was started at the same time as in the experiments in which nordihydroguaiaretic acid was infused. Samples were taken for the measurement of glucose, lactate, and pyruvate. Oxygen was monitored polarographically. Data are represented as changes relative to the values just before NAD^+ infusion and are the means ± mean standard errors of six to eight (control) or five to seven (nordihydroguaiaretic acid) liver perfusion experiments.

μM NAD^+, however, the changes were less pronounced than those observed with the concentration of 50 μM. In both absolute and relative terms, the effects on lactate production were smaller than those ones on glucose release. Pyruvate production also suffered an initial transitory inhibition with a subsequent recovery, but without significant stimulation relative to the basal values. At the end of the infusion of the highest NAD^+ concentrations (50 and 100 μM), there was a clear tendency toward inhibition. Some experiments were conducted in which the action of free nicotinamide was investigated. This compound, at a concentration of 100 μM, did not produce any alteration of the parameters investigated in the present study.

In order to find out if the metabolic action of NAD^+ is Ca^{2+} dependent, a series of experiments were done in which the cellular Ca^{2+} stores were depleted and the perfusion fluid was also Ca^{2+}-free. The Ca^{2+} depletion procedure was described in Materials and methods. Care was taken to avoid glycogen depletion during the procedure by adding 5 mM glucose, 2 mM lactate, and 0.2 mM pyruvate to the perfusion fluid. After the depletion procedure, a substrate-free perfusion fluid was again used, and the influence of 100 μM NAD^+ on the metabolic fluxes was evaluated. The mean basal rates of glucose release, oxygen consumption, lactate production, and pyruvate production under Ca^{2+}-free conditions were 0.87 ± 0.05, 1.97 ± 0.05, 1.00 ± 0.09, and 0.15 ± 0.02 μmol min^{-1} (g $liver)^{-1}$, respectively. Thus, they were not substantially different from those ones measured in the presence of Ca^{2+} (see preceding paragraph). Fig. 3 compares the response of the liver to 100 μM NAD^+ infusion in the absence of Ca^{2+} with that of the control condition. All NAD^+ effects were either diminished or almost completely eliminated. The latter is especially valid for glucose release and oxygen uptake. In the case of pyruvate production, a steady inhibition prevailed at the end of the infusion period.

A question that can be raised is if the response to NAD^+ can be restored if Ca^{2+} is reintroduced in the presence of the effector. Fig. 4 shows mean values of experiments in which the NAD^+ infusion was started after Ca^{2+} depletion by

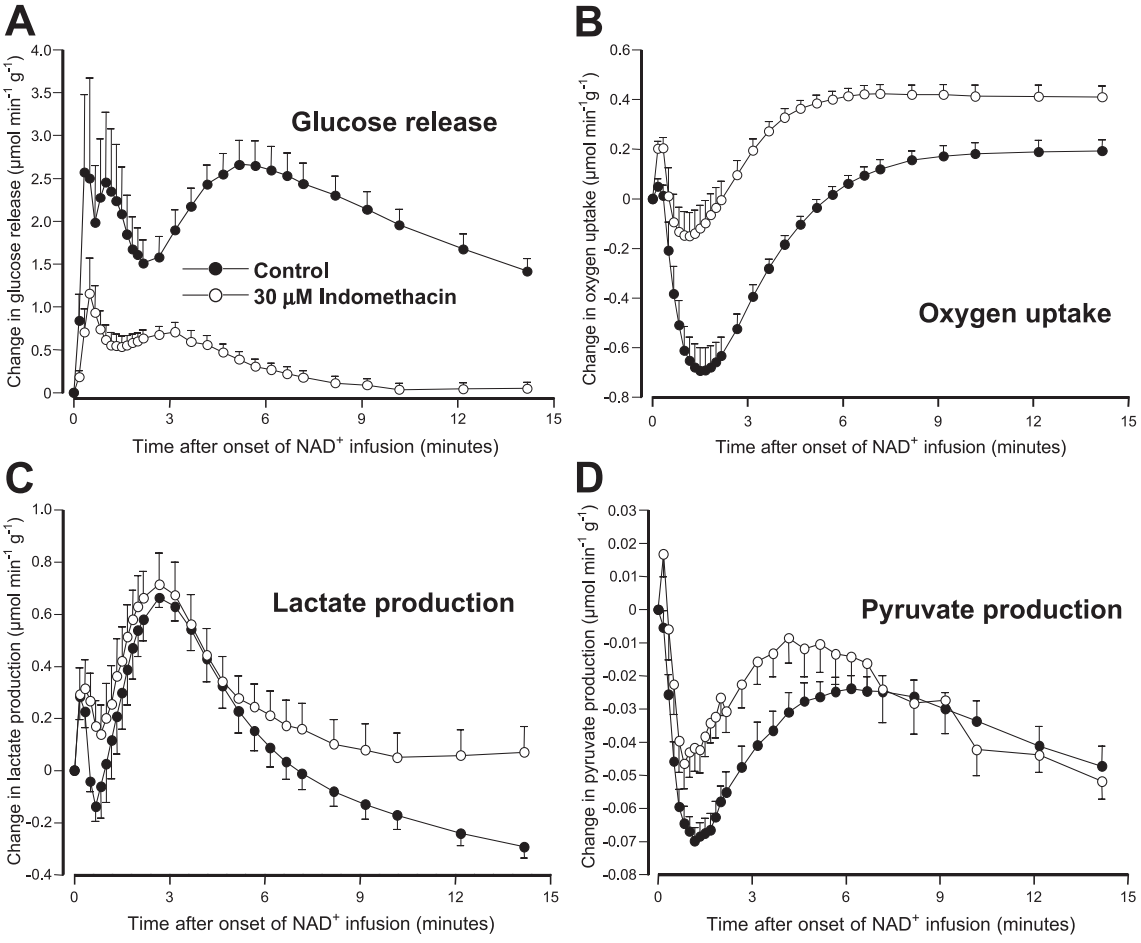

Fig. 8. Influence of indomethacin on the metabolic effects of NAD^+. Livers from fed rats were perfused as described in Materials and methods. Infusion of indomethacin (30 μM) was started 16 min prior to the NAD^+ infusion (100 μM) and continued until the end of the experiment. In the control experiments, NAD^+ infusion was started at the same time as in the experiments in which indomethacin was infused. Samples were taken for the measurement of glucose, lactate, and pyruvate. Oxygen was monitored polarographically. Data are represented as changes relative to the values just before NAD^+ infusion and are the means ± mean standard errors of six to eight (control) or five (indomethacin) liver perfusion experiments.

means of the same procedure employed in the experiments shown in Fig. 3. As expected, the responses of all metabolic parameters to NAD^+ were minimal, but the full responses were elicited when Ca^{2+} was introduced at 40 min perfusion time. These results illustrate again the Ca^{2+} dependence of the NAD^+. Furthermore, these results also exclude the possibility that the differences observed in the experiments illustrated by Fig. 3 could be caused by glycogen depletion.

Many Ca^{2+}-dependent agonists are also active on hemodynamics. In order to explore this possibility, a pressure transducer was used for measuring the portal perfusion pressure. The results are shown in Fig. 5. The introduction of NAD^+ transiently increased the portal perfusion pressure by approximately 12 mm Hg. The peak time of this pressure increase is similar to the peak time of oxygen uptake inhibition (compare Figs. 3 and 5). When Ca^{2+} was omitted from the perfusion fluid, the pressure increment was almost eliminated.

In order to investigate the possible participation of eicosanoids in the action of NAD^+, experiments were done with three inhibitors of eicosanoid synthesis. Bromophenacyl bromide is an inhibitor of phospholipase A_2 (Volwerk et al., 1974), which catalyzes the first step of eicosanoid synthesis. The introduction of this compound alone did not produce significant changes in metabolic fluxes. It was also inactive when glycogenolysis was stimulated by glucagon. With 10 nM glucagon, glycogenolysis in livers from fed rats was equal to 3.48 ± 0.36 μmol min^{-1} g^{-1}, and with glucagon + bromophenacyl bromide, a mean glucose release rate of 3.22 ± 0.43 μmol min^{-1} g^{-1} was measured. When NAD^+ was introduced in its presence, however, the response of glucose release, oxygen consumption, and lactate production were strongly reduced, as illustrated by Fig. 6. The reduction was similar to that one found with Ca^{2+}-free perfusion (compare Figs. 3 and 6). The action of NAD^+ on pyruvate production (panel D), however, was partly attenuated (initial inhibition) and partly modified (a stronger tendency toward inhibition at the end of the infusion).

Nordihydroguaiaretic acid is an inhibitor of leukotriene synthesis (Chang et al., 1984). Its effects on the basal rates was also minimal. However, in its presence, the effects of NAD^+ were substantially modified, as illustrated by Fig. 7. The extent of the first burst of glucose release stimulation was reduced by 80%; the second burst was reduced by 50% and its peak time delayed by several minutes (panel A). Inhibition of oxygen uptake, on the other hand, was clearly increased and prolonged (panel B): after 22 min NAD^+ infusion time, oxygen uptake was still inhibited, whereas in the control condition recovery from inhibition occurred around the eighth minute of NAD^+ infusion. Stimulation of lactate production was abolished (panel C); actually, the effect of NAD^+ was turned into a small and somewhat complex inhibition. The inhibition of pyruvate production (panel D), finally, was strongly increased by nordihydroguaiaretic acid.

Indomethacin is an inhibitor of prostaglandin, thromboxane, and prostacyclin synthesis (Vane, 1971). As shown by Fig. 8, it also substantially diminished the action of NAD^+ on glucose release (panel A) and oxygen uptake (panel B). It caused only a limited diminution of the NAD^+ effects on pyruvate production (panel D), and it was almost without effect on the action of NAD^+ on lactate production (panel C).

4. Discussion

The results of this work indicate that extracellular NAD^+ is able (a) to induce Ca^{2+} efflux from the liver cells, (b) to affect hepatic metabolism, especially glycogen breakdown, and (c) to increase the portal perfusion pressure. The last two effects are Ca^{2+} dependent, i.e., they were no longer present when this cation was absent, but could be restored when the cation was reintroduced. All these characteristics are typical of Ca^{2+}-dependent agonists, including those ones which are able to act directly on hepatocytes, as norepinephrine, for example (Reinhart et al., 1982; Yamamoto et al., 1992). However, there is an additional set of observations suggesting that the action of NAD^+ observed in the present study is actually mediated by eicosanoids. This set of observations comprises the sensitivity of the metabolic effects of NAD^+ to three substances which are amply recognized as inhibitors of eicosanoid synthesis, which are bromophenacyl bromide (Volwerk et al., 1974), nordihydroguaiaretic acid (Chang et al., 1984), and indomethacin (Vane, 1971). The action of these substances was generally inhibition, especially bromophenacyl bromide, an inhibitor of phospholipase A_2 which catalyzes the first reaction of the pathway leading to eicosanoid synthesis. In both qualitative and quantitative terms, the action of bromophenacyl bromide was very similar to the effect of Ca^{2+}-free perfusion, an observation that is consistent with the Ca^{2+} dependence of phospholipase A_2 (Birmelin et al., 1984). The only effect of NAD^+ which is likely to be independent of eicosanoid production is the second-phase pyruvate production inhibition which also occurs in the absence of Ca^{2+} and in the presence of bromophenacylbromide, which should suppress very strongly eicosanoid synthesis in general (Volwerk et al., 1974).

The conclusion that the effects of NAD^+ that were found in the present work are mediated by eicosanoids receives substantial support from investigations in which the action of eicosanoids in the perfused liver were examined. Prostaglandins increase Ca^{2+} release, glucose production, and portal perfusion pressure (Altin and Bygrave, 1988). The compound ONO-11113, an analog of thromboxane A2, diminishes oxygen consumption and increases glucose production and perfusion pressure and stimulates Ca^{2+} efflux. These effects are largely Ca^{2+} dependent (Altin and Bygrave, 1988). Similar effects have been reported for leukotrienes C4 and D4, which are able to increase glucose release and perfusion pressure and decrease oxygen consumption (Häussinger et al., 1988). Excepting thus the

changes in lactate and pyruvate production, for which no data are available in the literature, the effects that were observed in the present study are consistent with the capabilities of the various eicosanoids. Evidently, if two or more different eicosanoids are released, the effects can be correspondingly complex, especially if they act synergistically or even antagonistically. These complexities are especially evident in glucose release stimulation, which was clearly a biphasic phenomenon, whereas the reported effects of eicosanoids are rather monophasic (Häussinger et al., 1988; Altin and Bygrave, 1988). Another indication that the final effects result from a combination of several mediators is the action of nordihydroguaiaretic acid: it reduces considerably the first peak of glucose release, retards and diminishes the second peak, and increases both inhibition of oxygen uptake and pyruvate production. Nordihydroguaiaretic acid inhibits leukotriene synthesis, and it is difficult to devise a simple interpretation for such divergent phenomena as diminution of glucose release stimulation and increase in oxygen uptake inhibition. For oxygen uptake inhibition, the most simple explanation would be that the route which is inhibited by nordihdyroguaiaretic acid (leukotriene synthesis) produces the effector which reverses inhibition of oxygen uptake, whereas the route that is inhibited by indomethacin (prostaglandins and thromboxanes) produces the main inhibitor. In consequence, inhibition should be enhanced in the presence of nordihydroguaiaretic acid. The increased initial pyruvate production inhibition would then be a simple consequence of the increased oxygen uptake inhibition, which tends to shift the cytosolic NAD^+–NADH redox potential to a more reduced state. Lactate production stimulation is possibly caused by leukotrienes, because it is strongly inhibited by nordihydroguaiaretic acid, which inhibits leukotriene production, but not inhibited by indomethacin, which does not affect leukotriene production. For glucose production stimulation, however, it is difficult to devise a simple explanation in as much as it presents a complex kinetics.

An observation that corroborates the view that NAD^+ is able to act via eicosanoid production is the observation of Tsiamitas et al. (1979) that NAD^+ stimulates prostaglandin biosynthesis in guinea pig myometrium. Although not referring specifically to the liver, this observation indicates that stimulation of prostaglandin synthesis by NAD^+ is possible. It must be added that the action of NAD^+ via eicosanoids also means that cell-to-cell interactions are a necessary event. In fact, eicosanoid synthesis in the liver is generally believed to occur mainly in Kupffer cells, the target cells being mainly hepatocytes (metabolism) and endothelial cells (hemodynamics). In this respect, the observation of Amar-Costesec et al. (1985) is perhaps highly significant. These authors found that the NAD^+-glycohydrolase activity is 65-fold higher at the periphery of Kupffer cells than at the periphery of hepatocytes. This supports the view of a cell-to-cell interaction because it proves that NAD^+ can be rapidly transformed at the surface of Kupffer cells. Synthesis of eicosanoids in Kupffer cells has been amply studied, and it may be stimulated by several agents including adenosine and nucleotides such as ATP and UTP (Häussinger et al., 1988).

It is not possible to infer from the available data if NAD^+ itself triggers the actions observed in the present work or if this is caused by one of its metabolites. There is presently a general tendency in the specialized literature of attributing the signalling function of NAD^+ to its product cyclic ADP-ribose (Lee, 1994; Ziegler, 2000; Koshiyama et al., 1991). It should be noted that cyclic ADP-ribose permeates the cell membrane very slowly. It has rapid access to the cellular space only when produced extracellularly from NAD^+ by the ADP-ribosyl cyclase, when added to permeabilized cells (Lilly and Gollan, 1995; Podestá et al., 2000) or when microinjected (Hirata et al., 2002). Another possibility would be ADP-ribose, which is the predominant product of extracellular NAD^+ hydrolysis in the perfused liver, as shown by Liersch et al. (1971). The action of cyclic ADP-ribose on the induction of Ca^{2+} efflux in hepatocytes, however, has already been examined with negative results (Lilly and Gollan, 1995; Hirata et al., 2002). This is evidently a finding in conformity with the proposition of an action mediated by eicosanoids from Kupffer cells. On the other hand, little is known about the action of ADP-ribose, and one should not exclude the possibility that this compound contributes in some way to the multiple effects that were observed in the present work. There is evidence indicating that ADP-ribose is not able to affect Ca^{2+} release in mammalian cells where cyclic ADP-ribose is clearly active (Yusufi et al., 2001), but there are also suggestions that ADP-ribose may be able to increase calcium transport efficiency (Perraud et al., 2001; Bastide et al., 2002). In addition to ADP-ribose, Liersch et al. (1971) have also found AMP at concentrations which were only 6% of those of ADP-ribose. Even for a 100% transformation of 100 μM NAD^+ on a single passage through the liver, an improbable event, this would correspond to only 6 μM AMP. This is a very low concentration, which cannot be responsible for the pronounced effects of 100 μM NAD^+, as can be inferred from previous measurements of the metabolic effects of AMP (Minguetti-Câmara et al., 1998). Adenosine, another compound which also affects liver metabolism (Fernandes et al., 1999), was not detected by Liersch et al. (1971), although it is certainly formed and rapidly taken up by the liver cells. When added together, the effects of adenosine and AMP resulting from NAD^+ transformation could thus be responsible for only a very small part of the total effects. Since nicotinamide was also inactive on the parameters investigated in the present study, future attentions should be concentrated on NAD^+, cyclic ADP-ribose, and ADP-ribose.

It still remains to be established under what conditions NAD^+ can be released from cells in vivo in order to exert paracrine actions. It is generally believed that the controlled release of NAD^+ by connexin can cause extracellular NAD^+ concentrations to rise locally (Franco et al., 2001; Bruzzone

et al., 2001; Ohlrogge et al., 2002). Since it has been demonstrated that extracellular NAD^+ is able to enhance cell proliferation (Franco et al., 2001), it is also believed that bursts of high local extracellular NAD^+ levels can occur in consequence of cell lysis in areas of inflammation and tissue injury (Ohlrogge et al., 2002). In the present stage of knowledge, it is very difficult to devise connections between these suggested roles for extracellular NAD^+ and the phenomena that were observed in the present work. However, the fact that the liver responds to extracellular NAD^+ is unlikely to be without any physiological meaning and should deserve attention in future investigations.

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