

Original Article

Transformation and action of extracellular NAD⁺ in perfused rat and mouse livers

Ana Carla BROETTO-BIAZON, Fabrício BRACHT, Livia BRACHT, Ana Maria KELMER-BRACHT, Adelar BRACHT*

Laboratory of Liver Metabolism, University of Maringá, 87020900 Maringá, Brazil

Aim: Transformation and possible metabolic effects of extracellular NAD⁺ were investigated in the livers of mice (*Mus musculus*; Swiss strain) and rats (*Rattus norvegicus*; Holtzman and Wistar strains).

Methods: The livers were perfused in an open system using oxygen-saturated Krebs/Henseleit-bicarbonate buffer (pH 7.4) as the perfusion fluid. The transformation of NAD⁺ was monitored using high-performance liquid chromatography.

Results: In the mouse liver, the single-pass metabolism of 100 µmol/L NAD⁺ was almost complete; ADP-ribose and nicotinamide were the main products in the outflowing perfusate. In the livers of both Holtzman and Wistar rats, the main transformation products were ADP-ribose, uric acid and nicotinamide; significant amounts of inosine and AMP were also identified. On a weight basis, the transformation of NAD⁺ was more efficient in the mouse liver. In the rat liver, 100 µmol/L NAD⁺ transiently inhibited gluconeogenesis and oxygen uptake. Inhibition was followed by a transient stimulation. Inhibition was more pronounced in the Wistar strain and stimulation was more pronounced in the Holtzman strain. In the mouse liver, no clear effects on gluconeogenesis and oxygen uptake were found even at 500 µmol/L NAD⁺.

Conclusion: It can be concluded that the functions of extracellular NAD⁺ are species-dependent and that observations in one species are strictly valid for that species. Interspecies extrapolations should thus be made very carefully. Actually, even variants of the same species can demonstrate considerably different responses.

Keywords: extracellular NAD; perfused mouse liver; perfused rat liver; transformation; metabolic effects

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Introduction

It is long been known that extracellular NAD⁺ can be rapidly hydrolyzed to ADP-ribose in isolated perfused rat livers^[1]. The enzymatic system responsible for the transformation of extracellular NAD⁺ or NADP⁺ is usually referred to as glycohydrolase/ADP-ribosyl cyclase^[2]. It has also been shown that rabbit and rat liver extracts are able to catalyze the formation of cyclic ADP-ribose^[3,4], and that the hepatic ADP-ribosyl cyclase activity is similar to that of other tissues. There are claims that the NAD glycohydrolase/ADP-ribosyl cyclase activity corresponds to the CD38 protein, which is present in many cells^[2]. The existence of CD38 in the plasma membrane of hepatocytes has indeed been demonstrated by Khoo and Chang^[5]. In liver cells, this protein shows a 10-fold higher specific activity in the sinusoidal membrane fraction than in the bile canalicular membrane fraction.

Recent investigations have shown that the main products in the effluent perfusate in the liver of Wistar rats after portal infusion of NAD⁺ are ADP-ribose, uric acid and inosine^[6].

Extracellular nucleotides such as NAD⁺ are considered to be ideally suited as extracellular signal transmitters because they can be rapidly mobilized from intracellular stores and the signal is rapidly terminated by degradation by nucleotide catabolizing enzymes. NAD⁺ is supposed to be preferentially released from intracellular stores in conditions of cell stress or inflammation^[7]. There are many cells that respond in several ways to extracellular NAD⁺. In perfused livers from Wistar rats (*Rattus norvegicus*), the infusion of NAD⁺ produces increases in portal and arterial pressures and the initial inhibition of oxygen consumption followed by stimulation, increase in glycogenolysis and inhibition of gluconeogenesis^[8,9]. These effects are Ca²⁺-dependent and seem to result from an interaction between parenchymal and non-parenchymal cells *via* eicosanoid production. Furthermore, recent evidence indicates that these metabolic effects of NAD⁺ in the liver are partly due to its transforma-

* Correspondence to Prof Adelar BRACHT.

E-mail adebracht@uol.com.br

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tion products, such as ADP-ribose, and that these effects are mediated by purinergic receptors^[6].

Unlike the rat liver, little is known about the action and transformation of NAD⁺ in the mouse liver. The NAD glycohydrolase/ADP-ribosyl cyclase is also present in mice^[10, 11], but no systematic study has been done in which transformation and the possible metabolic effects of extracellular NAD⁺ in the mouse liver have been investigated. To fill this gap, transformation of NAD⁺ was investigated using high-performance liquid chromatography (HPLC). In addition, gluconeogenesis from lactate and its associated changes in oxygen uptake were measured as typical NAD⁺-sensitive metabolic parameters. In order to accentuate these comparative characteristics, experiments were done with isolated perfused mouse livers and perfused livers from two rat strains, namely, Wistar and Holtzman. Since NAD⁺ has been proposed to be a universal extracellular signal transmitter and since there are many suggestions about its future use as a therapeutic drug^[2, 7], it is also of interest to characterize its transformation and effects in the greatest possible number of species.

Materials and methods

Materials The liver perfusion apparatus was built in the workshops of the University of Maringá. NAD⁺ and all enzymes and coenzymes used in the enzymatic assays and chromatographic standards were purchased from Sigma Chemical Co (St Louis, USA). All standard chemicals were of the best available grade (98%–99.8% purity).

Animals and liver perfusion Male Swiss mice (*Mus musculus*), weighing 30–40 g, were fed *ad libitum* with a standard laboratory diet (Nuvilab®). Male albino rats (*Rattus norvegicus*; Wistar and Holtzman strains), weighing 200–250 g, were fed *ad libitum* with the same laboratory diet. Food was withdrawn 24 h prior to the perfusion experiments. All experiments were done in accordance with the worldwide accepted ethical guidelines for animal experimentation. For the surgical procedure, rats and mice were anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg). *In situ* hemoglobin-free, non-recirculating perfusion was performed^[12] with cannulation of both portal and cava veins. The perfusate flow was provided by a peristaltic pump (Minipuls 3, Gilson, France) and was around 12 mL·min⁻¹ for the mouse liver and around 31 mL·min⁻¹ for the rat liver. 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 Krebs/Henseleit-bicarbonate buffer was composed of 115 mmol/L

NaCl, 25 mmol/L NaHCO₃, 5.8 mmol/L KCl, 1.2 mmol/L Na₂SO₄, 1.18 mmol/L MgCl₂, 1.2 mmol/L NaH₂PO₄ and 2.5 mmol/L CaCl₂.

Analytical methods NAD⁺ and its metabolites in the outflowing perfusate (Krebs/Henseleit-bicarbonate buffer, pH 7.4) were separated by means of high-performance liquid chromatography (HPLC). The HPLC system (Shimadzu, Japan) consisted of an SCL-10AVP system controller, two model LC10ADVP pumps, a model CTO-10AVP column oven and a model SPD-10AVP UV-VIS detector. Standards were dissolved in Krebs/Henseleit-bicarbonate buffer, pH 7.4. Solutions were filtered through disposable 0.2 µm filters directly before injection. A reversed-phase C18 HRC-ODS column (5 µm; 150 × 6 mmol/L ID; Shimadzu, Japan) protected with a GHRC-ODS pre-column (5 µm; 10×4 mm ID; Shimadzu, Japan) was used with a gradient from reversed-phase 0.044 mol/L phosphate buffer solution, pH 6.0, to 0.044 mol/L phosphate buffer solution plus methanol (1.1), pH 7.0, at 0.8 mL·min⁻¹. The gradient consisted of the following steps (in % of methanol): 0 min, 0%; 2.5 min, 0.5%; 5 min, 3%; 7 min, 5%; 8 min, 12%; 10 min, 15%; 12 min, 20%; 20 min, 30%. The temperature was kept at 35 °C, and the injection volume was constant at 20 µL. The UV-absorbance detector was autozeroed at the start of each chromatogram, and absorbance was measured at 254 nm.

For the identification of the components in the perfusate, a retention time window of ±2% was adopted. The concentrations of NAD⁺ and its transformation products in each perfusate sample were calculated by means of the regression parameters obtained from calibration curves. The calibration curves were constructed by chromatographically separating standard Krebs/Henseleit-bicarbonate buffer solutions of the various compounds. Linear relationships were obtained between the concentration and the areas under the absorbance curves. The mean parameters obtained for the calibration curves were the following: (a) NAD⁺, linear regression coefficient=17214±98, correlation coefficient=0.999; (b) nicotinamide, linear regression coefficient=2922±82, correlation coefficient=0.999; (c) ADP-ribose, linear regression coefficient=14350±196, correlation coefficient=0.999; (d) AMP, linear regression coefficient=12222±347, correlation coefficient=0.999; (e) inosine, linear regression coefficient=14904±94, correlation coefficient=0.999; (f) uric acid, linear regression coefficient=4400±11, correlation coefficient=0.999.

During metabolic analyses of NAD⁺, the oxygen concentration in the effluent perfusate was monitored continuously using a teflon-shielded platinum electrode^[12]. Samples of the effluent perfusion fluid were collected according to the

experimental protocol and analyzed for glucose content^[13]. The metabolic rates were expressed as μmol per minute per gram liver wet weight ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$).

Data analysis The metabolic rates were expressed as μmol per minute per gram liver wet weight ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$). Statistical analysis of the data was done by means of the Statistica™ program (Statsoft®, 1998). Student's *t* test was applied for evaluating the statistical difference between two means; $P < 0.05$ was adopted as a criterion of significance. Peak times and times for half-maximal recovery of glucose production and oxygen uptake during NAD^+ infusion of livers from Wistar and Holtzman rats were determined using numerical interpolation (Stineman's interpolation) by means of the Scientist® software from MicroMath Scientific Software (Salt Lake City, UT, USA).

Results

Transformation of extracellular NAD^+ in mouse and rat livers

Single-pass transformation of extracellular NAD^+ was measured. After a short pre-perfusion period in which oxygen uptake by the liver was allowed to stabilize, NAD^+ was infused. Samples of the outflowing perfusate were collected for chromatographic analyses at different times after starting the infusion. In the mouse liver perfusion, two different NAD^+ concentrations were infused, 100 and 500 $\mu\text{mol/L}$. In the mouse liver, the single-pass extraction of NAD^+ was practically complete when the compound was infused into the portal vein at the concentration of 100 $\mu\text{mol/L}$ (Figure 1, curve A). At 500 $\mu\text{mol/L}$, however, some NAD^+ was recovered (Figure 1, curve A). In addition to the non-transformed NAD^+ , two transformation products were found, ADP-ribose and nicotinamide, as can be deduced from their retention times, 9.54 ± 0.06 and 17.37 ± 0.11 min, respectively. Very small amounts of uric acid (5.51 ± 0.05 min) and AMP (12.98 ± 0.01 min) were also produced and were clearly visible in the chromatogram of the 100 $\mu\text{mol/L}$ NAD^+ infusion (Figure 1, curve A). Livers from both rat strains, Wistar (curve A) and Holtzman (curve B), show similar transformation products (Figure 2). At least six components could be identified: uric acid (5.51 ± 0.05 min), ADP-ribose (9.54 ± 0.06 min), AMP (12.98 ± 0.01 min), NAD^+ (16.70 ± 0.06 min), nicotinamide (17.37 ± 0.11) and inosine (17.91 ± 0.12 min). AMP was also clearly visible for the Holtzman strain. The first peak, with a retention time of around 3.5 min, could not be identified. No transformation product with a retention time longer than 20 min was found, an observation that excludes free adenosine, which had a retention time of 23.64 ± 0.07 min. Furthermore, cyclic-ADP-

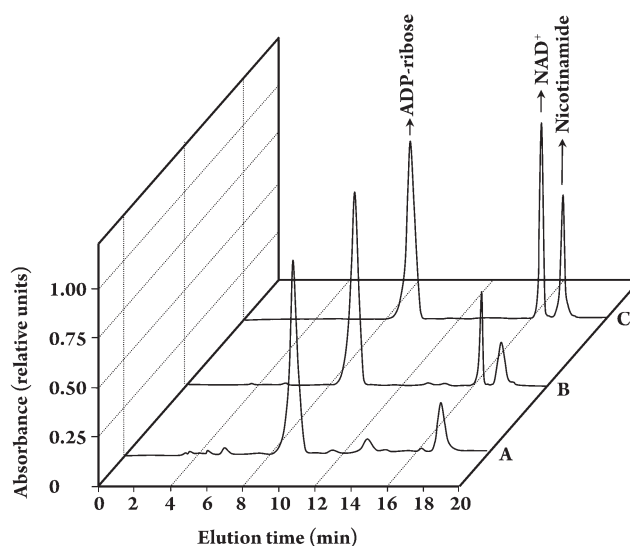


Figure 1. Typical chromatographic profiles of standards and outflowing per-fusate of the mouse liver. Perfusate samples were collected at 1.3 min after initiation of NAD^+ infusion. HPLC fractionation with spectrophotometric detection at 254 nm was done as described in the Materials and methods section. Legends: (A) outflowing perfusate with 100 $\mu\text{mol/L}$ NAD^+ infusion; (B) outflowing perfusate with 500 $\mu\text{mol/L}$ NAD^+ infusion; (C) standards. The absorbance scale was normalized as a fraction of the highest absorbance peak in each run.

ribose (cADPR) and β -nicotinic acid adenine dinucleotide phosphate (β -NAADP) were expected to appear with retention times between 7 and 8 min^[6]. This region, however, was practically empty in all chromatograms obtained in the present work (Figures 1 and 2).

The concentrations of all identified compounds in the effluent perfusate were calculated from the areas under the chromatographic curves using the regression coefficients obtained from appropriate calibration curves, as described in the Materials and Methods section. These concentrations were represented as a function of the NAD^+ infusion times. For the mouse liver, the portal 100 $\mu\text{mol/L}$ NAD^+ infusion corresponds to an infusion rate of $0.8 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$. Since metabolism of NAD^+ was complete under these conditions, $0.8 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ also corresponds to the transformation rate. The concentration of ADP-ribose in the outflowing perfusate increased during the first 3 min of NAD^+ infusion and stabilized thereafter (Figure 3). The concentration of nicotinamide also increased during the first 3 min and reached approximately 90% of the NAD^+ input (Figure 3). ADP-ribose and nicotinamide, however, were not released in equimolar amounts, suggesting a more accentuated intracellular processing of the ADP-ribose moiety of NAD^+ . The

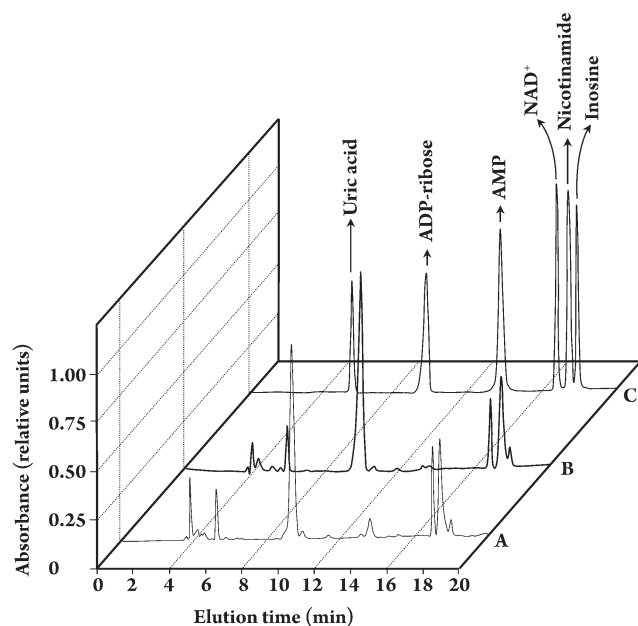


Figure 2. Typical chromatographic profiles of standards and outflowing perfusate of rat livers from the Wistar and Holzman strains. Perfusate samples were collected at 1.3 min after initiation of 100 $\mu\text{mol/L}$ NAD^+ infusion. HPLC fractionation with spectrophotometric detection at 254 nm was done as described in the Materials and methods section. Legends: (A) Holzman strain; (B) Wistar strain; (C) standards. The absorbance scale was normalized as a fraction of the highest absorbance peak in each run.

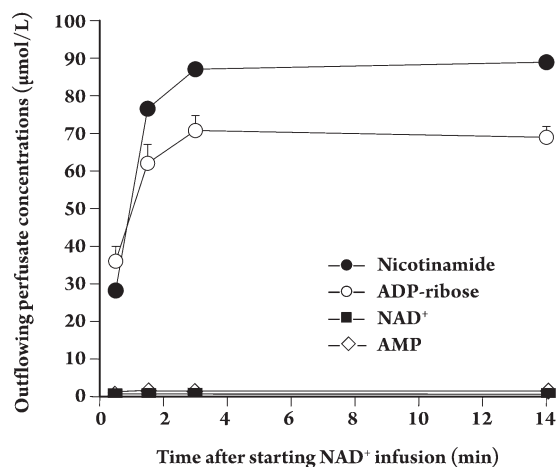


Figure 3. Time course of 100 $\mu\text{mol/L}$ NAD^+ transformation in the perfused mouse liver. Livers of fasted mice were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in Materials and Methods. The concentrations of NAD^+ and its metabolites in the outflowing perfusate at different times after initiation of NAD^+ infusion were determined by HPLC. Data are the means \pm mean standard errors of four liver perfusion experiments.

productions of AMP and uric acid were minimal. In the mouse liver, the portal 500 $\mu\text{mol/L}$ NAD^+ infusion corresponds to an infusion rate of 4 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$. Under these conditions, ADP-ribose and nicotinamide were released in almost equimolar amounts (Figure 4). A decline in the concentration of both ADP-ribose and nicotinamide was evident at 14 min of infusion. This decline was accompanied by an increase in NAD^+ recovery, which reached 28.5% at 14 min. The rate of NAD^+ transformation at this time was 2.86 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$. It seems, therefore, that the capacity of NAD^+ transformation decreased significantly with the infusion time, at least at this high portal concentration of 500 $\mu\text{mol/L}$. Here again, the productions of AMP and uric acid were very small.

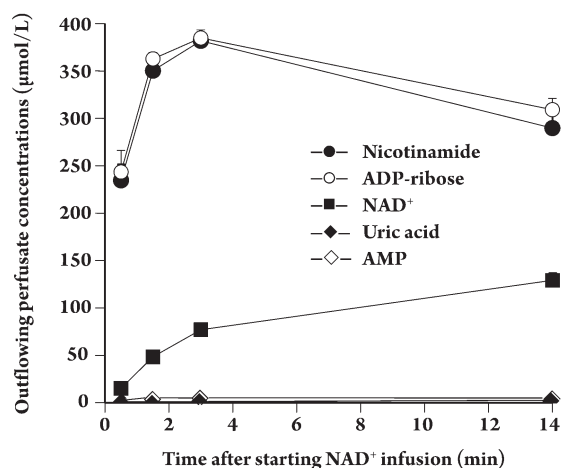


Figure 4. Time course of 500 $\mu\text{mol/L}$ NAD^+ transformation in the perfused mouse liver. Livers of fasted mice were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in Materials and Methods. The concentrations of NAD^+ and its metabolites in the outflowing perfusate at different times after initiation of NAD^+ infusion were determined by HPLC. Data are the means \pm mean standard errors of four liver perfusion experiments.

In the rat liver, a portal NAD^+ concentration of 100 $\mu\text{mol/L}$ corresponds to an infusion rate of 0.32 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$. The single-pass transformation of NAD^+ was 97% at all infusion times, except at 1.5 min, where it was 94% (Figure 5). An extraction of 97% corresponds to a transformation rate of 0.31 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$. A similar, but considerably higher, recovery peak was also found in livers from rats of the Wistar strain, and it coincided with the time of maximal increase in perfusion pressure caused by NAD^+ ^[6]. Nicotinamide tended to approach 90% of the infused NAD^+ at the end of the infusion period (Figure 5). AMP and inosine

comprised very small fractions of the transformation products, but the contributions of ADP-ribose and uric acid were highly significant (Figure 5). This is again partly similar to what was found in livers from the Wistar strain, but in those rats, ADP-ribose was considerably more abundant than uric acid, especially at the end of the infusion period^[6].

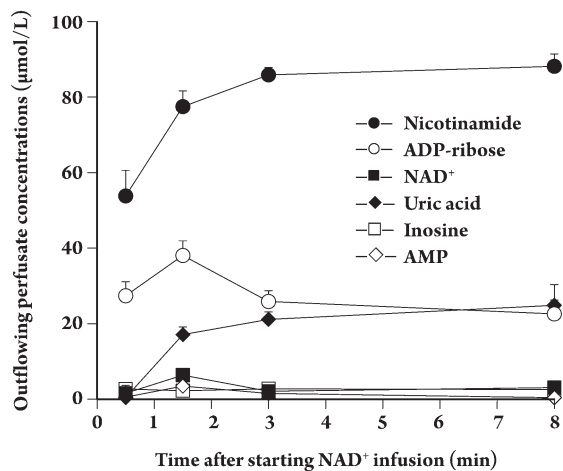


Figure 5. Time course of 100 $\mu\text{mol/L}$ NAD^+ transformation in the perfused liver of Holtzman rats. Livers of fasted rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in Materials and Methods. The concentrations of NAD^+ and its metabolites in the outflowing perfusate at different times after initiation of NAD^+ infusion were determined by HPLC. Data are the means \pm mean standard errors of four liver perfusion experiments.

Metabolic actions of extracellular NAD^+ in mouse and rat livers In the liver of the Wistar rats, NAD^+ and its major transformation product, ADP-ribose, are able to induce transient decreases in both oxygen uptake and gluconeogenesis from lactate (Figure 6)^[6,9]. Livers from rats of the Holtzman strain also respond in this way (Figure 7). In the experiments, livers from 24-h fasted rats were perfused in order to minimize interference by glycogen catabolism^[9]. Lactate (2 mmol/L) was infused for 44 min before a 100 $\mu\text{mol/L}$ NAD^+ infusion (Figures 6 and 7). At 46 min, both oxygen uptake and gluconeogenesis had almost stabilized. The NAD^+ induced rapid decreases in both oxygen uptake and gluconeogenesis, followed by recoveries that culminated in transient stimulations. The peak decreases and stimulations found in livers of Wistar and Holtzman rats were evaluated. It is apparent that the peak inhibitions of gluconeogenesis and oxygen uptake were more pronounced (by factors of 2.4 and 2.8, respectively) in the Wistar strain (Table 1). The opposite occurred with the transient stimulations, which were more pronounced in the livers of the Holtzman strain

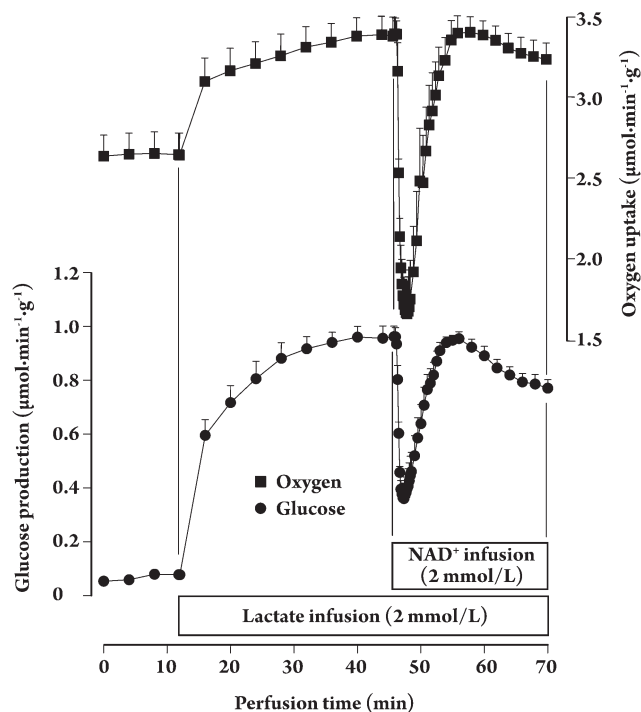


Figure 6. Gluconeogenesis and oxygen uptake in the liver of Wistar rats: influence of lactate and NAD^+ . Livers from 24-h fasted rats were perfused as described in Materials and Methods. After oxygen uptake stabilization, lactate and NAD^+ (at the given concentrations) were infused at the indicated times. Samples of the effluent perfusate were collected for glucose assay. Oxygen was monitored polarographically. Data represent the means \pm SEM of five liver perfusion experiments.

(by factors of 6.0 and 4.3, respectively, for gluconeogenesis and oxygen uptake). The metabolic effects of NAD^+ were further characterized by evaluating the times for maximal inhibition (peak times) of glucose production and oxygen uptake and the times for half-maximal recoveries ($t_{1/2}$). These parameters have been considered measures of the persistency of the metabolic effects of extracellular NAD^+ ^[14]. In livers from Holtzman rats, the peak times are approximately half those of livers from Wistar rats (Table 2). Additionally, the recovery is 2.95 to 4.59 times faster in livers from rats of the Holtzman strain (Table 2).

To test how the mouse liver behaves during NAD^+ infusion, similar perfusion experiments were done with livers from fasted mice (Figure 8). The infusion of lactate increased both glucose production and oxygen uptake to extents similar to the rat liver. The infusion of 100 $\mu\text{mol/L}$ NAD^+ , on the other hand, produced only small alterations in both glucose production and oxygen uptake (Figure 7 and Table 1). The standard errors are quite high, denoting poor significance. Repetition of the experiments with 500 $\mu\text{mol/L}$

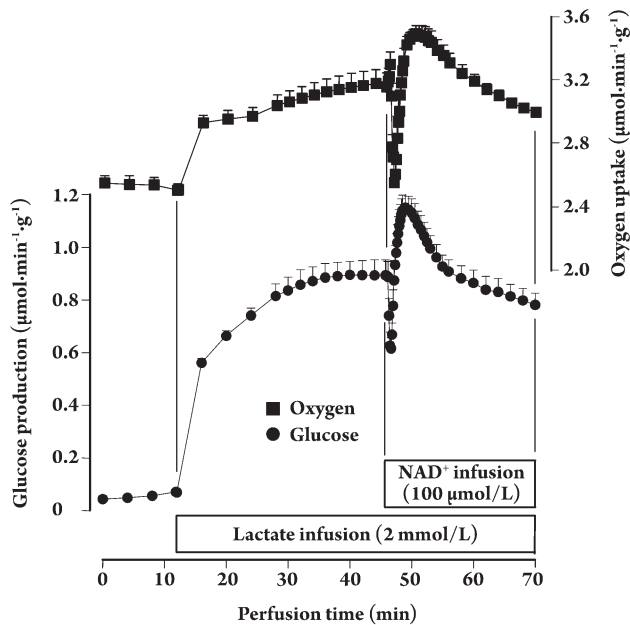


Figure 7. Gluconeogenesis and oxygen uptake in the liver of Holtzman rats: influence of lactate and NAD^+ . Livers from 24-h fasted rats were perfused as described in Materials and Methods. After oxygen uptake stabilization, lactate and NAD^+ (at the given concentrations) were infused at the indicated times. Samples of the effluent perfusate were collected for glucose assay. Oxygen was monitored polarographically. Data represent the means \pm SEM of five liver perfusion experiments.

NAD^+ was unsuccessful: no significant enhancements of the metabolic responses were found with this concentration (data not shown). The mouse liver also did not respond to a 100 $\mu\text{mol/L}$ ADP-ribose infusion (data not shown), an expected phenomenon if one considers that a high concentration of this metabolite is generated during a 500 $\mu\text{mol/L}$ NAD^+ infusion.

Discussion

The results of the present work extend previous knowl-

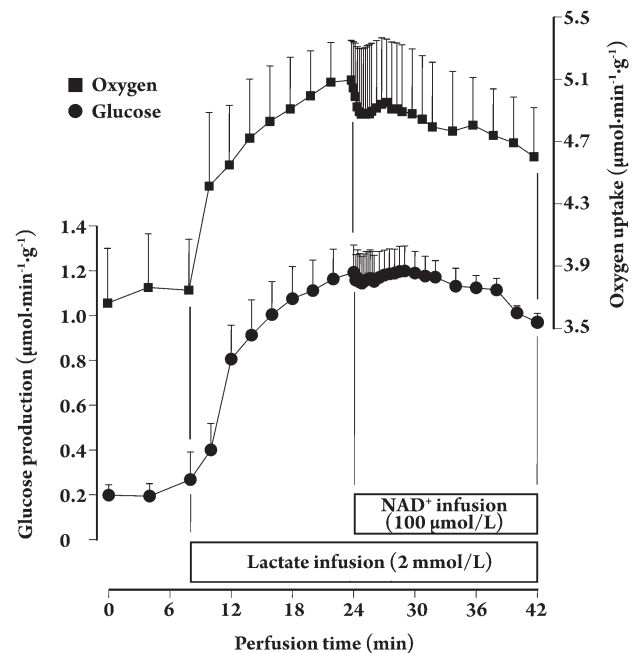


Figure 8. Gluconeogenesis and oxygen uptake in the mouse liver: influence of lactate and NAD^+ . Livers from 24-h fasted mice were perfused as described in Materials and Methods. After oxygen uptake stabilization, lactate and NAD^+ (at the given concentrations) were infused at the indicated times. Samples of the effluent perfusate were collected for glucose assay. Oxygen was monitored polarographically. Data represent the means \pm mean standard errors of five liver perfusion experiments.

edge about the transformation and effects of extracellular NAD^+ in the livers of Wistar rats as compared to the livers of Holtzman rats and mice. The mouse liver possesses a large capacity for metabolizing extracellular NAD^+ into basically two products: ADP-ribose and nicotinamide. Actually, the mouse liver is more efficient than the rat liver when the transformation rates per unit liver weight are compared. The weight of the mouse livers used in the present work corresponded to approximately 15% of the weight of the

Table 1. Peak decreases and peak stimulations in glucose production and oxygen uptake during NAD^+ infusion in livers from Holtzman rats, Wistar rats and Swiss mice. The data were obtained from perfusion experiments of the kind illustrated by Figures 6 and 7. Peak inhibition represents the difference between the rate just before the onset of NAD^+ infusion and the minimal rate immediately after initiation of the infusion. Similarly, peak stimulation represents the difference between the rate just before the onset of NAD^+ infusion and the maximal rate after the recovery from inhibition. The *P* values refer to Student's *t* test in the comparison between the Holtzman and Wistar strain. ^a*P*=0.001; ^b*P*=0.002; ^c*P*=0.004; ^d*P*=0.01.

Animal	Peak inhibition ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$)		Peak stimulation ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$)	
	Glucose production	Oxygen uptake	Glucose production	Oxygen uptake
Rat, Holtzman (<i>n</i> =5)	0.291 \pm 0.042 ^a	0.631 \pm 0.158 ^b	0.284 \pm 0.047 ^c	0.364 \pm 0.058 ^d
Rat, Wistar (<i>n</i> =4)	0.692 \pm 0.069 ^a	1.770 \pm 0.180 ^b	0.047 \pm 0.020 ^c	0.084 \pm 0.053 ^d
Mouse, Swiss (<i>n</i> =5)	0.067 \pm 0.038	0.222 \pm 0.202	–	–

Table 2. Peak times and times for half-maximal recovery in glucose production, and oxygen uptake during NAD⁺ infusion in livers from rats of the Holtzman and Wistar strains. The data were obtained from perfusion experiments of the kind illustrated by Figure 6. The *P* values refer to Student's *t* test in the comparison between the Holtzman and Wistar strain. ^a*P*=0.003; ^b*P*<0.001; ^c*P*=0.001; ^d*P*=0.004.

Strain	Peak time (min)		Time for half-maximal recovery, <i>t</i> _{1/2} (min)	
	Glucose production	Oxygen uptake	Glucose production	Oxygen uptake
Holtzman (<i>n</i> =5)	0.67±0.05 ^a	1.05±0.03 ^b	0.62±0.08 ^c	0.83±0.15 ^d
Wistar (<i>n</i> =4)	1.21±0.12 ^a	1.98±0.10 ^b	2.85±0.32 ^c	2.45±0.40 ^d

rat livers, and metabolism of the 100 μmol/L NAD⁺ portal infusion was complete in the former but not necessarily in the latter^[6]. It should be remarked that the lower metabolism of 500 μmol/L NAD⁺ when compared to 100 μmol/L NAD⁺ reflects, most likely, the fact that the transformation is a saturable process like all enzyme-catalyzed processes. The fact that only two products could be detected in significant amounts in the outflowing perfusate in the mouse liver perfusion presents a noteworthy contrast to the rat liver perfusion, in which several additional transformation products were detected, especially uric acid and inosine. This is true for both the Wistar^[6] and Holtzman strains. ADP-ribose and nicotinamide can be generated extracellularly, but uric acid and inosine result from reactions within the cell. The virtual absence of uric acid and inosine in the effluent perfusate in the mouse liver perfusion could thus reflect differences in intracellular transformation of the extracellular NAD⁺ products between mice and rats.

It should be noted that cyclic-ADP-ribose, an important signaling molecule that originates from extracellular NAD⁺ transformation, was not found in the outflowing perfusate in both mouse and rat liver perfusions. This observation, however, does not exclude its production. In both mouse and rat livers, cyclic-ADP-ribose is probably produced in very small amounts compared to ADP-ribose – only one molecule of cADP-ribose is generated intracellularly for every 100 molecules of NAD⁺ that are hydrolyzed^[15]. Furthermore, cADP-ribose probably does not permeate the cell membrane, so that it cannot be expected to appear in the outflowing perfusate. These cells probably comprise a minor fraction of the total hepatic cell population. It has been reported that, at least in the rat liver, the NAD⁺-glycohydrolase activity is 65-fold higher in the plasma membrane of Kupffer cells than in the plasma membrane of hepatocytes^[16]. This indeed makes it difficult to detect cyclic-ADPR in the whole liver, because Kupffer cells comprise a relatively small fraction of the total cell population in the liver.

The metabolic responses to NAD⁺ in livers from the Wistar and Holtzman strains clearly indicate stronger inhibi-

tion in the former and a more pronounced stimulation in the latter. These observations, however, do not necessarily mean that the signals generating inhibition and stimulation are different in the two strains. The times for maximal inhibition and the times for half-maximal recovery are both considerably shorter in the Holtzman strain. This observation could be indicating that only the stimulation is actually faster in the Holtzman strain. At least in theory, a faster stimulation with a similar rate of inhibition in the Holtzman strain would explain the different responses found in the present work.

The rapid transformation of NAD⁺ in the mouse liver contrasts with the absence of effect on oxygen uptake and gluconeogenesis. This does not necessarily mean that NAD⁺ is metabolically inert in the mouse liver. Gluconeogenesis and the associated oxygen uptake are only two among many metabolic pathways that could be affected by the compound. However, the fact that gluconeogenesis and oxygen uptake in the rat liver respond intensely to NAD⁺^[6,9] allows at least the conclusion that observations in one species are only valid for that species and that generalizations and interspecies extrapolations should be done very carefully. Actually, differences can occur even between different strains of the same species, which is evident from the comparison of the results obtained in the present work with livers from rats of the Holtzman strain with those of the Wistar strain that were reported previously^[6,8]. The differences include both the proportions of transformation products and the metabolic effects. The transformation products are equally active in metabolism, *e.g.*, ADP-ribose and inosine^[6], and the differences in the metabolic effects could be partly due to this phenomenon. One should remember that the functions of extracellular NAD⁺ or its main transformation products depend on eicosanoid synthesis^[6,9]. In this respect, there are a few reports in the literature about different responses of Wistar and Holtzman rats to agents whose action also depends on eicosanoid synthesis. It is well known, for example, that, in the induction of arthritis, Holtzman rats respond in a stable way to Freund's adjuvant^[17], whereas the response of Wistar rats varies considerably among individuals^[18]. Another

example is the action of bradykinin, which induces dose-dependent contraction of relaxed mesenteric arterial rings from Holtzman rats, but not from Wistar rats, and is mediated by eicosanoids^[19]. Experiments on platelet aggregation induced by arachidonic acid, a precursor of eicosanoid synthesis, can also be regarded as an indicator that Holtzman rats may respond differently when eicosanoids are involved. Platelets from the Holtzman strain were more sensitive to arachidonic acid than were those from other strains^[20]. Concerning the very pronounced difference in the response to extracellular NAD⁺ between mice and rats, there are no hints in the literature that this could be used to generate a working hypothesis, so this is a question that remains entirely open to future investigations. It is appropriate to remember, on the other hand, that there is currently intense investigation worldwide aiming to elucidate the precise roles of extracellular NAD⁺ in mammals, but that the ultimate scope is to define its role in humans^[2, 7, 21]. In this respect, the accentuated differences found in the present work between rats and mice, and even between different rat strains, emphasize the necessity to perform more studies on the action of extracellular NAD⁺ in humans – or at least in cells derived from humans.

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Author contribution

Dr Adelar BRACHT and Dr Ana Maria KELMER-BRACHT designed the research, analyzed the data and wrote the article; Dr Ana Carla BROETTO-BIAZON and M Sc Livia BRACHT performed the liver perfusion experiments and analyzed the data; Fabricio BRACHT was responsible for the chromatographic analyses.

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