The role of adrenergic agonists on glycogenolysis in rat hepatocyte culture and possible involving of NO.

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Short title: Adrenergic agonists in glycogenolysis and NO

Summary: Certain liver metabolic diseases point to the presence of disturbances in glycogen deposition. Epinephrine raises the cAMP level that activates protein kinase A leading to the activation of phosphorylase and glycogen breakdown. In the present work, we sought to investigate whether NO is produced during adrenoceptor agonist- induced glycogenolysis in rat hepatocytes in cultures. Isolated glycogen rich rat hepatocytes in cultures were used. NO production (NO_2^-) was assessed under the effect of adrenergic agonists and adrenergic agonist/antagonist pairs, dibutyryl cyclic AMP sodium-potassium salt (db-cAMP), the NO

synthase (NOS) inhibitors N^{ω}-nitro-L-arginine methyl ester (L-NAME), aminoguanidine (AG), the NO donor S-nitroso-N-acetyl penicillamine (SNAP). The inducible NO synthase (iNOS) mRNA was examined by reverse transcription-polymerase chain reaction (RT-PCR). Glycogenolysis was quantified by glucose levels released into medium. The amount of glucose and NO_2^- released by hepatocytes was increased as a result of epinephrine, phenylephrine or db-cAMP treatments. The increase in glucose and NO_2^- released by epinephrine or phenylephrine was blocked or reduced by prazocin pretreatment and by NOS inhibitors aminoguanidine and Nω-nitro-L-arginine methyl ester (L-NAME). iNOS gene expression was up-regulated by epinephrine. It can be concluded, that glycogenolysis occurs through α-adrenoceptor stimulation and signaling cascade may involve NO production.

Introduction: Liver disturbances which occur in certain metabolic diseases may point to the presence of disturbances in glycogen deposition due to various reasons that either promote accumulation of glycogen in the liver or lead to depressed levels of liver glycogen where the two processes are reciprocally controlled. Control of hepatic glycogenolysis involves intricate paracrine and endocrine communication between hepatocytes and other cells (Borgs et al. 1996, Decker 1990, Huber and Keppler 1990). Signaling pathways that are included in hormone-induced glycogen degradation are well documented. Glucagon is known to be the most potent activator of glycogenolysis and its role on glycogenolysis has been extensively studied. In addition, epinephrine raises the cAMP level that activates protein kinase A (PKA) and this in turn leads to the activation of phosphorylase and glycogen breakdown. At the same time, PKA phosphorylates and helps to inactivate glycogen synthase. This means that the latter two

hormones on the one hand, and insulin on the other, determine which one predominates. This helps to ensure that when glycogen is breaking down it is not also synthesized; the controls are reciprocal on the two systems (Elliott and Elliott 2001). The adrenergic regulation of glycogenolysis in hepatocytes is of importance under both physiological and pathological conditions. A number of studies were conducted in this regard where alpha 1 and beta receptors were reported to exist in hepatocytes (Van Ermen and Fraeyman 1994, Shiroyama et al. 1998, Fabbri et al. 1999, Vardanega-Peicher et al. 2000, Manzl et al. 2002). The reported data indicate that several types of adrenergic receptors may play role in glycogenolysis. Even a change from beta- to alpha-adrenergic glycogenolysis in rat liver was reported (Moriyama et al. 1997).

Though the pathways that modulate glycogen level in the liver are almost clear, the role of nitric oxide (NO) in this process is not extensively studied. Currently it is well known that NO possesses functional regulatory effects in all organs, tissues and cells which were examined including the liver. The role of NO in a number of liver functions has been investigated in previous studies (Muriel 2000, Chang et al. 2004). It was suggested that nitric oxide synthases (NOSs) are new players in the pathophysiology of some liver diseases (Moreau 2002). Reports indicate that NO affects glycogen and glucose homeostasis (Borgs et al. 1996, Stadler et al. 1995, Horton et al. 1994 (1), Horton et al.1994 (2), Sugita et al. 2002). However, it is still unclear how NO is involved in the modulation of glycogenolytic pathways or in liver glycogen homeostasis. Modulation of reactions involved in production and release of glucose from hepatocytes is a potential approach to develop new classes of oral hypoglycemic agents (Proietto and Andrikopoulos 2004). In the present work, the fact whether NO is produced during adrenoreceptor agonist- induced glucose release in rat hepatocyte in cultures was investigated. To achieve this goal, isolated glycogen-rich rat hepatocytes in culture were used for

glycogenolysis studies and NO expression was assessed under the effect of various adrenergic agonists, adrenergic agonist/antagonist pairs, cAMP and nitric oxide donors.

Key words: glycogen, glycogenolysis, NO, iNOS, epinephrine, cAMP

Methods:

Chemicals and reagents - William′s medium E, supplemented with gentamicin, L-glutamine and 10% FBS, bovine serum albumin fraction V (BSA), epinephrine, phenylephrine, prazocin, propranolol (+/- 1-isopropylamino-3-(1-naphthyloxy)-2-propranolon hydrochloride), sulfanilic acid, N-(1-Naphtyl)ethylendiamine dihydrochloride, dibutyryl cyclic AMP sodium-potassium salt (db-cAMP), N^{ω}-nitro-L-arginine methyl ester (L-NAME), aminoguanidine (AG), S-nitroso-N-acetyl penicillamine (SNAP), dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Prague). Collagenase and Glucose assay kit weres obtained from Sevapharma (Prague). All other chemicals were obtained from standard sources and were the highest grade available.

Animals, isolation and preparation of glycogen rich hepatocyte culture: Male rats of Wistar strain (Velaz - Lysolaje, 200-250 g body weight) were used throughout the present studies. Hepatocytes were isolated from rats by the standard two-phase perfusion method where collagenase was included in the second phase as previously reported (Farghali et al 1994). After isolation, cell viability as assessed by trypan blue was more than 90%. The preparation of glycogen rich hepatocytes was carried out according to Shiroyama et al. 1998 with modification. Briefly, 1.5×10^6 hepatocytes from untreated animals were plated into 35-mm collagen-coated cell culture dishes and maintained at 37^0C, 95% air, and 5% CO_2 in William′s medium E, supplemented with gentamicin, L-glutamine 10 mM HEPES, 6% fetal bovine

serum, 10^{-7} M insulin and 20 mM glucose. The glycogen rich cells were used to determine glycogenolytic glucose release. High glycogen content of hepatocytes was confirmed by both biochemical and histochemical means (Shiroyama et al. 1998). This model proved to be convenient for the study of glycogenolysis.

Measurement of glucose release- The medium of the glycogen rich hepatocytes was changed to Dulbecco's phosphate buffered saline (containing neither glucose nor amino acids, nor lactate) just before the appropriate treatment. Control hepatocyte cultures without agonist addition were always included to express the precise glycogenolytic effect of agonists. Epinephrine, (10^{-7} -10^{-4} M), phenylephrine (10^{-7} -10^{-4} M), isoprenaline (2 μM), dobutamine (10 μM) without or preceded with prazocin or propranolol to the glucose-free Dulbecco's phosphate buffered saline medium. The time course of glycogenolysis was followed till 120 min of incubation. Glycogenolysis was followed up by estimation of medium glucose medium levels spectrophotometricaly according to an enzymatic glucose assay kit.

Nitric oxide production and other biochemical measurements - The time course of NO production, cell protein content (protein kit Sigma-Aldrich, Czech Republic), ALT leakage (Biocon, Germany) from cells into the medium were measured in appropriate time intervals as indicated in the section of results. NO production was determined by measuring the levels of its oxidation products NO_2^- and NO_3^- in the culture medium. This was detected colormetrically (540 nm) by Griess reagent. The nitrite levels were extrapolated from $NaNO_2$ calibration curve.

iNOS expression in cultured hepatocytes by RT-PCR - The 24-h cultured hepatocytes were used for reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from hepatocytes was isolated by the standard procedure described earlier (Farghali et al. 2002) for

iNOS. The relative level of iNOS mRNA expression was determined after normalization to the β-actin signal to account for variability in the amount of RNA that had been extracted from cells.

Statistical analysis - All experiments were performed at a minimum 6-8 times in triplicates (means of at least of 18-24 values, see results) with blind samples as the media background. The statistical significance of difference of mean scores was determined using the unpaired Student's t-test. Results showing p-values less than 0.05 were considered statistically significant.

Results

The amount of glucose and nitrite released by glycogen-rich hepatocytes was significantly increased as a function of time by adrenergic drugs treatment. Figure 1 demonstrates that at sampling times 30 and 60 min, both glucose and nitrite levels were higher under adrenergic drugs treatment as compared to control treatment. Nitrite level was also higher in both cases as compared to control which was statistically significant in 60 min after incubation of hepatocyte in culture. The increase in the amount of glucose and nitrite induced by epinephrine or phenylephrine was completely blocked or significantly reduced by prazocin pretreatment, respectively as evidenced from Figure 2. The last Figure also demonstrates that the pretreatment with propranolol has no effect on epinephrine-induced increase in glucose neither in nitrite production. In hepatocyte culture, treatment with epinephrine increased iNOS gene expression as measured by RT-PCR. Figure 3 shows a representative photograph of an agarose gel of iNOS mRNA levels. In non-induced, control hepatocytes, iNOS mRNA was detected. Following treatment with epinephrine there was an intense PCR product of iNOS that was observed after 60 min exposure of hepatocyte in culture. At posttranslational level, Table 1 shows the relative effect

of incubation (30 and 60 min) with 5 treatments on nitrite production and glycogenolysis in hepatocyte culture after 24 h. Both aminoguanidine at high concentrations (10 mM) and L-NAME (10 mM) were able to significantly decrease NO production and glycogenolysis induced by epinephrine. This effect was measurable and significant in most cases at 30, 60 min with aminoguanidine being more potent.

Table 1. The relative effect of incubation (30 and 60 min) with 5 treatments on nitrite production and glycogenolysis in hepatocyte culture after 24 h expressed in % of control (mean values of 20-24 culture ± SEM)

Treatments	Percent of control ± SEM**			
	Nitrite after		Glucose after	
	30 min	60 min	30 min	60 min
AG	78±7*	38±8*	91±15	90±13
AG+Epi	83±3*	79±6*	103±15	158±13*
NAME	90±10	78±9*	100±3	140±3*
NAME+Epi	158±7*	78±6	126±13	93±15
db-cAMP	120±10*	160±8*	140±10*	90±25

AG- aminoguanidine, **Epi**- epinephrine, **NAME**- N^{ω}-nitro-L-arginine methyl ester ,
db-cAMP - dibutyryl cyclic AMP sodium-potassium salt
* significant difference from control $P \leq 0.05$.
**percent of the amount of produced nitrite or glucose in treated culture per the amount in control culture

Discussion: The liver plays a central role in the control of glucose production. Due to the importance of hepatocytes in this regard, intensive research is directed towards finding out pharmacological means that modulates hepatocyte signaling pathways leading to glycogenolysis

or modulation of gluconeogenesis. This important aspect may be directed to patients with type 2 diabetes who are going on to require insulin therapy to achieve glucose control. New classes of oral hypoglycemic agents could be targeting the inappropriately elevated endogenous glucose production. Among several approaches reported (Moriyama et al. 1997) are modulators of glycogenolysis and gluconeogenesis, inhibitors of stimulatory hormones or their receptors. The present data demonstrate that epinephrine and phenylephrine but not isoprenaline or dobutamine caused stimulation of glucose release in hepatocytes in culture. However, reports indicate that various adrenergic, both alpha and beta agonists, stimulate glycogenolysis depending on animal age and zones of hepatocytes (Fraeyman and Van Ermen 1993, Sanghani and Scarpace 1994, Tosh and Agius 1994).

Nevertheless the present study demonstrates that NO is released, probably, as a common denominator signal during the process of glycogenolysis as revealed for both adrenergic-agonistic effect with consequent glucose release. Measurement of glycogenolysis was carried out through the preparation of glycogen rich hepatocytes according to Shiroyama et al. 1998 which were used to determine glycogenolytic glucose release in glucose-free incubation medium. The incubation of hepatocytes in William's medium E containing high glucose and insulin concentrations (to get glycogen rich hepatocytes) and the switching into Dulbecco's phosphate buffered saline medium should turn down to minimum the expected pathway of gluconeogenesis which is based mainly on lactate as a reactant (Meyer et al.2003). Dulbecco's phosphate buffered saline medium as the only medium in the second part of experiment disables additional pathways of forming glucose based on uptake of reaction substrates from extra-cellular enviroment.

The role of nitric oxide (NO) in a number of liver functions has been investigated in several previous studies. It was suggested that nitric oxide synthases (NOSs) are players in the

pathophysiology of some hepatic diseases (Moreau 2002). In several other reports it was found that NO affects glycogen and glucose homeostasis (Borgs et al. 1996, Stadler et al. 1995, Horton et al. 1994 (1), Horton et al. 1994 (2), Sugita et al. 2002). However, it is still unclear how NO is involved in the modulation of glycogenolytic pathways under physiological conditions.

Interesting enough, it was found in this work that the α_1-selective adrenoceptor blocker (prazocin) inhibited epinephrine-induced glycogenolysis and NO production as well.
The present data demonstrates evidences that α–adrenergic -induced glycogenolysis which, is realized via the agonist/adenylyl cyclase/protein kinase A (PKA) cascade and cAMP signaling pathway, involve downstream NO production. This is supported by the fact that the stable congener to cAMP, db-cAMP, produced identical effects on glycogenolysis and NO production to epinephrine. Moreover, the used SNAP at the concentrations of 25-250 μM in hepatocyte culture led into dose-dependent increase in basal glycogenolysis (data not shown). Therefore,
in our study it can be suggested that endogenous NO produced downstream of agonist/receptor signal transduction pathway and coupling through an isoform of NOS plays a role in glycogenolysis. This is further supported by our data demonstrating that both L-NAME and aminoguanidine were able to partially inhibit the glycogenolytic effect of epinephrine. Moreover, iNOS mRNA was significantly enhanced by epinephrine. Indeed, the role of iNOS in –adrenoceptor - stimulated glycogenolysis under physiological conditions was not yet reported according to the available data. The present data do not rule out involvement of either NOS isoform in contributing to the glycogenolytic signaling pathway(s) in isolated cultured hepatocytes. Maintaining blood glucose levels within a physiological range is an important function requiring multiple metabolic pathways and involving several cell types, including the important role for hepatocytes. The importance of this study, therefore, stems out from the fact

that hepatocytes are critical for glucose homeostasis (Klover and Mooney 2004). Hepatocytes can respond to either feeding or fasting by storing or producing glucose as necessary. Glucagon, catecholamines and insulin are well studied regulators of glycogen stores. Transcriptional regulation of rate limiting enzymes and modulation of enzyme activity through phosphorylation and allosteric regulation are involved. Therefore, the contribution of more signals which are involved in these functions would be significant. Our data indicate that under the present experimental conditions, glycogenolysis occurs through α- adrenoreceptor stimulation, which is known to be realized via the adenylyl cyclase/cAMP protein kinase A signaling cascade may involve NO production downstream of receptor- cAMP pathways. It was reported that iNOS is regulated mainly at the transcriptional level by several transcriptional factors such as nuclear transcription factor- κB (NF-κB), Fos/Jun, CCAAT/enhancer binding protein (C/EBP), interferon-γ (IFN-γ) activation site (GAS), and IFN regulatory factor (IRF) and some of these transcription factors (AP-1, NF-κB, and C/EBP) are reported to be regulated by glycemia. Therefore, there is a possibility that glycogenolysis may be involved in the regulation of iNOS gene expression via the regulation of these transcriptional factors (Won et al. 2003).

This gives further proof about the diversity of NO (whether at physiological levels or the induced ones) roles in hepatocyte metabolic functions.

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Legend to Figures

Figure 1 (A, B)

Glucose (A) and nitrite (B) levels under epinephrine and phenylephrine treatments. 1) 30 min incubation control, 2) 30 min incubation epinephrine and phenylephrine, 3) 60 min incubation control, 4) 60 min incubation epinephrine and phenylephrine. * Significant different from control $P \leq 0.05$.

Figure 2. **(A, B)**

The effect of prazocin and propranolol pretreatment on glucose (A)- and nitrite (B) -induced increase by epinephrine and phenyephrine treatments. * Significant different from control $P \leq 0.05$.

Figure 3. A representative photograph of an agarose gel of iNOS mRNA levels.
1) control hepatocytes, 2) epinephrine, 3) epinephrine +L-NAME, 4) L-NAME.

Fig 1.

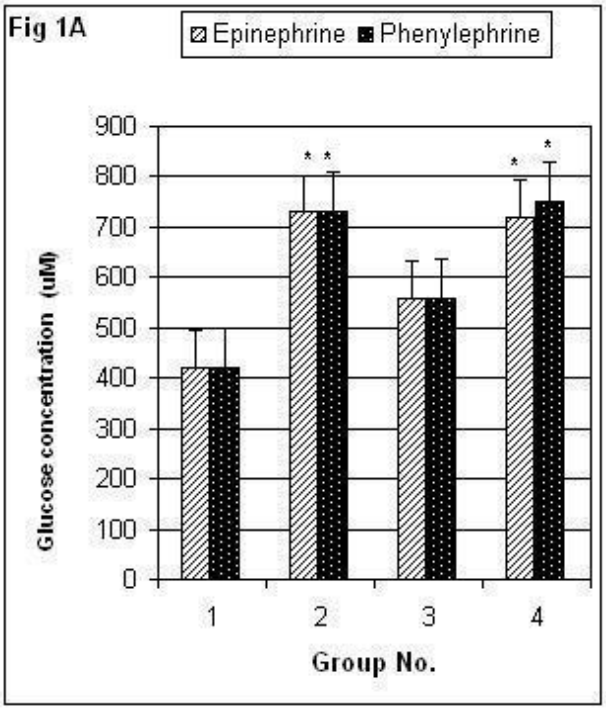
Fig 1A
Epinephrine
Phenylephrine
Glucose concentration (uM)
900
800
700
600
500
400
300
200
100
0
1
2
3
4
Group No.

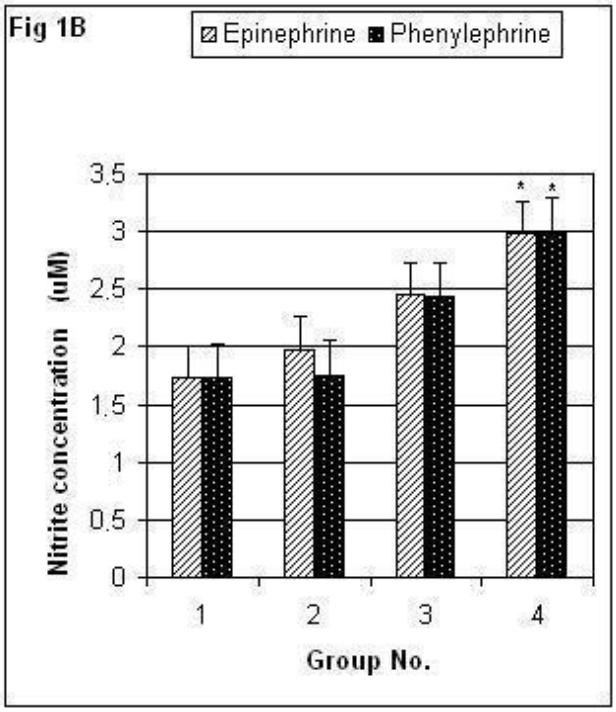
Fig 1B
Epinephrine
Phenylephrine
Nitrite concentration (uM)
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
Group No.

Fig 2.

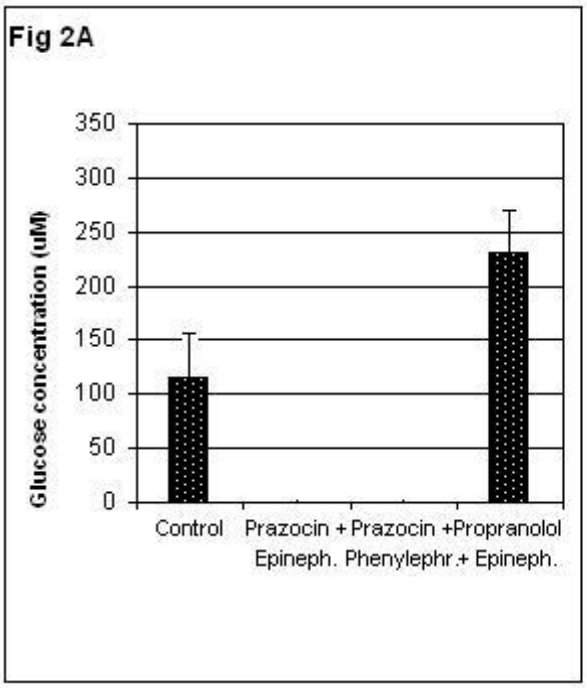
Fig 2A
Glucose concentration (uM)
350
300
250
200
150
100
50
0
Control
Prazocin + Epineph.
Prazocin + Phenylephr.
Propranolol + Epineph.

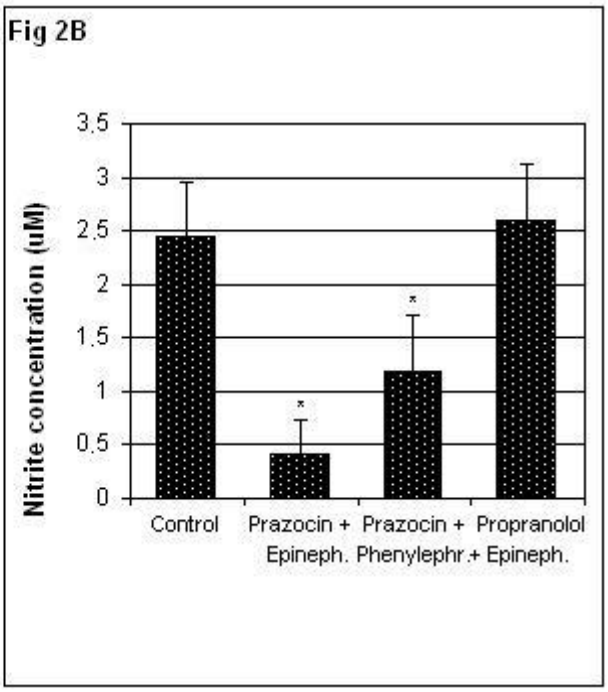
Fig 2B
Nitrite concentration (uM)
3.5
3
2.5
2
1.5
1
0.5
0
Control
Prazocin + Epineph.
Prazocin + Phenylephr.
Propranolol + Epineph.

Fig 3. iNOS- mRNA levels