Glucose release as a response to glucagon in rat hepatocyte culture: Involvement of NO signaling.

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Summary

Glucagon and α–adrenergic-induced glycogenolysis is realized via the agonist/adenylyl cyclase/cAMP/protein kinase signaling pathway or via the activation of phosphorylase kinase by the mobilized calcium that supports the inhibition of glycogen synthase, respectively. The role of nitric oxide (NO) in this process is not extensively studied. The present work was directed to the question whether NO is produced during glucagon-induced glycogenolysis in rat hepatocyte in a similar way like α-adrenoceptor stimulation. Glycogen-rich hepatocyte cultures were used. NO production (NO_2^-) was assessed under the effect of glucagon, dibutyryl cyclic AMP (db-cAMP), forskolin, the nitric oxide synthase (NOS) inhibitors N^{ω}-

nitro-L-arginine methyl ester (L-NAME) and aminoguanidine, and the NO donor S-nitroso-N-acetyl penicillamine (SNAP). Inducible NOS (iNOS) mRNA was examined by reverse transcription-polymerase chain reaction. Glycogenolysis was followed up by estimation of medium glucose levels. The amount of glucose and NO_2^- released by glycogen-rich hepatocytes was increased as a result of glucagon, db-cAMP, forskolin and SNAP treatments. iNOS gene expression was upregulated by glucagon. Glycogenolysis that occurs through glucagon receptor stimulation involves NO production downstream of transduction pathways through an isoform of NOSs. The present and previous studies document the possible involvement of NO signaling in glycogenolytic response to glucagon and adrenergic agonists in hepatocytes.

***Key words**:* nitric oxide; Glucagon; glycogenolysis; hepatocytes

Introduction

It is established that the liver plays a central role in the control of glucose production and that disturbances, which occur in some metabolic diseases, could indicate the presence of alterations in glycogen deposition of various reasons with either increase or decrease in liver glycogen. The molecular mechanisms of increased glucose production to identify potential therapeutic targets were reviewed (Proietto and Andrikopoulos 2004, Klover and Mooney 2004) and it has been recognized that hepatocytes are critical for glucose homeostasis. The signaling pathways that participate in hormone-induced glycogen degradation are documented (Borgs et al. 1996, Decker 1990, Huber and Keppler 1990).

Glucagon raises cyclic AMP (cAMP) levels that activate 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. Moreover, epinephrine through its

α_1-adrenergic receptors regulates hepatic glycogenolysis in some animal species. Altogether, glucagon and epinephrine on the one hand, and insulin on the other, determine the status of glycogen store. The regulation of glycogenolysis in hepatocytes by both hormones appears to be equally important under physiological and pathological conditions (Van Ermen and Fraeyman 1994, Shiroyama et al. 1998, Fabbri et al. 1999, Vardanega-Peicher et al.2000, Manzl et al.2002, Kalamidas et al. 2002, Moriyama et al. 1997).

Although the pathways that modulate glycogen level in the liver have been fairly well elucidated, the role of nitric oxide (NO) in this process is not extensively studied. In our previous work, we found that glycogenolysis in rat hepatocytes that occurs through α-adrenergic stimulation involve NO production (Hodis et al. 2007). Indeed, NO possesses functional regulatory effects in all organs, tissues and cells, which were examined including the liver (Chang et al. 2004, Muriel 2000, Moreau 2002, Voleti and Agrawal 2006, Harbrecht et al. 2004). Reports indicate that NO affects glycogen and glucose homeostasis (Borgs et al. 1996, Stadler et al. 1995, Horton et al. 1994_a, Horton et al. 1994_b, Sugita et al. 2002) and data suggest that glucagon may participate in the in vivo regulation of hepatic inducible nitric oxide synthase (iNOS) expression after proinflammatory stimuli. Nevertheless, it is not yet clear to which extent NO is involved in the modulation of glycogenolytic pathways. The present work was, therefore, directed to extend our previous observation by the study that shed more light on whether NO is produced during glucagon-induced glycogenolysis in rat hepatocyte cultures in a similar way like adrenergic stimulation (Hodis et al. 2007). The glycogen-rich rat hepatocytes in culture were used for glycogenolysis studies under glucagon, dibutyryl cyclic AMP (db-cAMP), forskolin, and the NO donor S-nitroso-N-acetyl penicillamine (SNAP). NO production was assessed under various treatments.

Materials and methods

Chemicals

Glucagon was obtained in ampoules (Glucagen 1 mg HypoKit inj. sic.) from Novo Nordisk, Denmark. 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, S-nitroso-N-acetyl penicillamine (SNAP), forskolin (7β-acetoxy-1α,6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one), dimethyl sulfoxide (DMSO), HEPES, Dulbecco's phosphate buffered saline, William′s medium E, gentamicin, L-glutamine and fetal bovine serum (FBS) and bovine serum albumin fraction V (BSA), were obtained from Sigma-Aldrich (Prague, Czech Republic). Collagenase and glucose assay kit were obtained from Sevapharma and Vian Diagnostika (Prague, Czech Republic), respectively. A commercial kit for nitrite/nitrate colorimetric detection was obtained from ROCHE Diagnostic (Prague). Other chemicals were obtained from standard sources.

Animals

Male rats of Wistar strain (Velaz-Lysolaje, 200-250 g body weight) received humane care and the study procedures were carried out in accordance with the local general guidelines of the 1st Faculty of Medicine.

Isolation and preparation of glycogen rich hepatocyte culture

Hepatocytes were isolated from anesthetized rats by a standard two-phase collagenase perfusion method as previously reported (Farghali et al. 1994). After isolation, cell viability was at least 90% as assessed by trypan blue. The preparation of glycogen-rich hepatocytes was performed according to Shiroyama et al. (Shiroyama et al.1998) with minor modification (Hodis et al. 2007). Hepatocytes from untreated animals were plated into collagen-coated cell

culture dishes and maintained at 37 °C, 95% air, and 5% CO_2 in William′s medium E, supplemented with gentamicin, L-glutamine, 10 mM HEPES, 6% FBS, $2x10^{-7}$ M insulin and 20 mM glucose overnight. The produced glycogen-rich cells were used to determine glycogenolyic glucose release. High glycogen content of hepatocytes was confirmed biochemically and histochemically (Shiroyama et al. 1998). This model proved to be convenient for the study of glycogenolysis by glucose release since there are no substrates as lactate, amino acids or other types of sugar necessary for gluconeogenesis.

Drug treatments and measurement of glycogenolytic glucose release

The medium of the glycogen-rich hepatocytes was changed to glucose free Dulbecco's phosphate buffered saline just before the relevant treatment. Glucagon (0.5-10 μg/ml) was added to the glucose-free Dulbecco's phosphate buffered saline medium in culture. Cultured hepatocytes were treated with forskolin (50 μM), db-cAMP (1 mM) or SNAP (25 and 250 μ M). DMSO at final concentration of less than 1% (v/v) used as the solvent for some compounds (e.g. forskolin) was found to be without any effect on the measured parameters. The time course of glucose release was followed till 60 min of incubation. The estimation of medium glucose levels was done spectrophotometricaly according to the enzymatic glucose assay kit instructions from Vian Diagnostika (Prague, Czech Republic).

Determination of nitric oxide production

NO production from the cells into the medium was 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 and/or by nitrite/nitrate colormetric method from ROCHE Diagnostic. The nitrite levels were extrapolated from $NaNO_2$ calibration curve.

Reverse transcriptase-polymerase chain reaction for iNOS expression

The 24-h cultured hepatocytes were used for reverse transcriptase-polymerase chain reaction (RT-PCR) under glucagon or without glucagon or L-NAME+glucagon treatment. RNA from hepatocytes was isolated by the standard procedure via Quiagen® RNeasy mini kit as described earlier for iNOS (Farghali et al. 2002). Total RNA content was finally measured via spectrophotometry-Eppendorf® BioPhotometer, RNA purity was counted from absorption rate of 260/280 nm respectively. The total RNA was amplified and reverse transcribed into cDNA via RT-PCR. 2.3 µl of cDNA formed was treated with PCR reaction mix after following protocol: Mix (47.7 µl) = H_2O 33.6 µl + red buffer 5.0 µl + black $MgCl_2$ 4.0 µl + primer F 0.3 µl + primer R 0.3 µl + blue NTP 1.0 µl + yellow enhancer 1.0 µl + Taq® polymerase 2.5 µl. Cycler´s conditions being: 2´94°//25x (20´´94°/10´´59°1 ´72°)//10´72°. The primers were constructed as described before [23]: F 5´-GGC AGA CTG GAT TTG GCT GGT C-3´; R 5´- AGG TGT TCC CCA GGT AGG TAG C-3´, comprising exon-intron DNA boundaries eliminating external contaminating DNA to be involved into PCR reaction. 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.

Amplified samples were run on agarose gels and stained with ethidium bromide. Images were captured using a monochrome camera and signal density measured by Kodak 1D Image Analysis Software Version 3.0 and compared to control sample.

Statistical analysis

In most of the experiments, the protein content of hepatocytes was estimated and was found to be variable per culture plate. Therefore, control culture plate was done for every single

experiment. The experiments were performed at a minimum 6-8 times in triplicates (means of 18-24 values ± SEM) with blind samples as the media background. The statistical significance of difference of mean scores was determined using the unpaired Student's t-test or ANOVA where appropriate. Results showing *P*-values equal to or less than 0.05 were considered statistically significant.

Results

Figure 1 demonstrates that the amount of glucose and nitrite released by glycogen-rich hepatocytes was significantly increased as a function of incubation time by glucagon (1 μ g/ml) treatment. These data show that at sampling times 30 and 60 min, both glucose and nitrite levels were higher under glucagon treatment as compared to control treatment. The effects were not evident at time 120 min (data are not shown). 30 and 60 min sampling times were, therefore, chosen for the subsequent experiments. In hepatocyte culture, treatment with glucagon increased iNOS gene expression as measured by RT-PCR. The expression was increased approximately twice according to data shown in Figure 2. The last Figure shows a photograph of an agarose gel of iNOS mRNA levels, where iNOS mRNA was detected in non-induced control hepatocytes. Following treatment with glucagon, there was an intense PCR product of iNOS that was observed after 60 min exposure of hepatocytes in culture. It is clear that L-NAME did not significantly affect the level of iNOS mRNA which is expected as L-NAME is an inhibitor of NOS per se (i.e. post-translation effect)

Table 1 shows the relative effect of incubation (30 and 60 min) with 5 treatments on nitrite production and glucose release in hepatocyte culture. Both aminoguanidine and L-NAME at high concentrations were able to significantly decrease NO production (measured as nitrite) and glycogenolysis (measured as glucose) induced by glucagon. This effect was measurable and significant in most cases at 30 and 60 min with aminoguanidine being more potent.

Moreover, Table 1 demonstrates that both db-cAMP and the specific activator of adenylyl cyclase, forskolin, significantly increased both glucose release and nitrite levels.

The effects of exogenously delivered NO (SNAP addition) on glucose release and nitrite levels is demonstrated in Figure 3. SNAP treatment, as expected, at 25 and 250 μM concentrations in the culture medium produced highly significant concentration-dependent increase in total nitrite levels as compared to control treatment (Fig. 3B). In addition, SNAP significantly increased basal glucose production 30 min after incubation when compared to control values (Fig. 3A).

Discussion

The role of NO in a number of liver functions is being investigated intensively. 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 not clear how NO is involved in the modulation of glycogenolytic pathways under physiological conditions. Moreover, there are data which indicate that glucagon may participate in the in vivo regulation of hepatic iNOS expression after proinflammatory stimuli (Harbrecht et al. 2004). The current investigation was motivated by our previous finding related to stimulation hepatocyte glycogenolysis after α-adrenoceptor stimulation with the involvement of NO release (Hodis et al. 2007). The importance of the present study stems from the fact that liver has a central role in the control of glucose production and that the hepatocytes are the main players. Therefore, research is directed towards finding out pharmacological means that modulates hepatocyte signaling pathways with consequent alterations in glycogenolysis or gluconeogenesis.

The present data demonstrate that glucagon-stimulated glycogenolysis in hepatocytes is accompanied by an increase in NO release. The present findings together with our previous ones (Hodis et al. 2007) demonstrate that NO is released, probably, as a common denominator signal during glycogenolysis as revealed for both α-adrenergic- and glucagon-agonistic effect.

The measurement of glycogenolysis was carried out through the preparation of glycogen-rich hepatocytes according to Shiroyama et al. (Shiroyama et al. 1998), which were used to determine glycogenolyic glucose release in glucose-free incubation medium. This was proved by the authors of the method (Shiroyama et al. 1998) and by our present results to be a suitable and direct informative method for measuring glycogenolytic effect of agonists. The higher nitrite levels produced by glucagon compared to control were, in most experiments, statistically significant 60 min after incubation of hepatocyte in culture. The effect of glucagon on NO production is expected to be evidenced at later time than glucose. The reason is due to the fact that it is not NO that is directly measured but rather its oxidation product in the medium i.e. nitrite/nitrate, which needs time to be formed from NO.

Taken together, it seems that glucagon- and α–adrenergic-induced glycogenlysis that are known to be realized via the agonist/adenylyl cyclase/PKA cascade and cAMP signaling pathway or via the activation of phosphorylase kinase by the mobilized calcium that is aided by the inhibition of glycogen synthase respectively (Dempsey et al. 2000) may involve downstream NO production. This is further evidenced by the adenylyl cyclase activator, forskolin, and the stable congener to cAMP, db-cAMP, that both of them produce qualitatively identical effects on glycogenlysis and NO production to glucagon. Moreover, we used SNAP at two concentrations added to hepatocyte culture, and we observed dose-dependent increase in basal glycogenlysis. Accordingly, we may suggest that endogenous NO produced downstream of agonist/receptor signal transduction pathway and coupling through an isoform of NOS, plays a role in glycogenolysis. These are further supported by our data demonstrating

that aminoguanidine and to some extent L-NAME were able to partially inhibit the glycogenolytic effect of glucagon and epinephrine. Moreover, iNOS mRNA was significantly enhanced by both glucagon (present data) and epinephrine (Hodis et al. 2007). The present data do not rule out the involvement of either NOS isoform in contributing to the glycogenolytic signaling pathway(s) in isolated cultured hepatocytes. Recently, we have found a similar involvement of NO signaling during β-adrenergic lipolysis in rat white adipocytes (Kutinova-Canova et al. 2006) indicating the wide spread role of NO in various pathophysiological processes of the liver as well as other well established functions in other organs and tissues.

In summary, our studies may suggest that under the present experimental conditions, endogenous NO is produced downstream of glucagon- receptor or α-adrenoceptor signal transduction pathways through an isoform of NOSs and that it plays a role in glocogenolysis. This is supported by the findings that: 1) glucagon and epinephrine increased NO and simultaneously stimulated glycogenolysis; 2) prazosin (a selective α-adrenergic blocker) but not propranolol (non selective β-adrenergic blocker) was able to inhibit the glycogenolytic effect of α-adrenergic agonists concomitantly with a reduction of the amount of nitrite formed in the medium; 3) both glucagon and epinephrine increased iNOS mRNA; 4) db-cAMP and the adenlyl cyclase activator, forskolin, stimulated both nitrite production and glucose release; 5) NO exogenously delivered through SNAP to hepatocyte culture stimulated glycogenolysis and; 6) inhibitors of NOSs reduced nitrite production and glycogenolysis in cultured rat hepatocytes. More studies, however, are needed to further clarify of NO role in glycogenolysis.

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Legend to Figures

Figure 1

Glucose **(A)** and nitrites **(B)** released to culture medium by glycogen-rich hepatocytes under control conditions and glucagon (1 μg/ml) treatment after 30 and 60 min. Values are expressed as means ± SEM. * Significantly different from the respective control group ($P \leq 0.05$).

Figure 2

A representative photograph of an agarose gel of RT-PCR demonstrating iNOS mRNA levels, where columns depict iNOS mRNA ladder with 386 bp and cultured hepatocytes under: 1) no treatment (control), 2) 1 μg/ml glucagon, 3) 1 μg/ml glucagon + 10 mM L-NAME treatment. The graph illustrates the quantified signal density of iNOS PCR products of treated hepatocytes compared to control sample. Results were recorded after 60 min incubation.

Figure 3

The effect of S-nitroso-N-acetyl penicillamine (SNAP, 25 and 250 μM) treatment on glucose **(A)** and nitrite **(B)** release from glycogen-rich hepatocytes cultured for 30 and 60 min. Values are expressed as means ± SEM. * Significantly different from the respective control group ($P \leq 0.05$).

Table 1

The relative effect of incubation (30 and 60 min) with glucagon (1 μg/ml), aminoguanidine (AG, 10 mM) + glucagons, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10 mM) + glucagon, dibutyryl cAMP (db-cAMP, 1 mM) and forskolin (50 μM) treatments on nitrite production and glucose release from hepatocytes in culture (mean values of 20-24 cultures ± SEM).

Treatments	Percent of control[a] ± SEM			
	Nitrite (μM) after		Glucose (μM) after	
	30 min	60 min	30 min	60 min
Glucagon	114±15	133±11*	267±25*	148±12*
AG + glucagon	75±5*	53±5*	90±15	117±10
L-NAME + glucagon	150±8*	76±6*	191±30*	150±15*
db-cAMP	120±10*	160±8*	140±10*	90±25
Forskolin	116±4*	120±3*	147±13*	75±8*

* Significantly different from control ($P \leq 0.05$).

[a] Percent of the amount of produced nitrite or glucose in treated culture per the amount in appropriate control culture (treated/control x 100).

Fig 1.

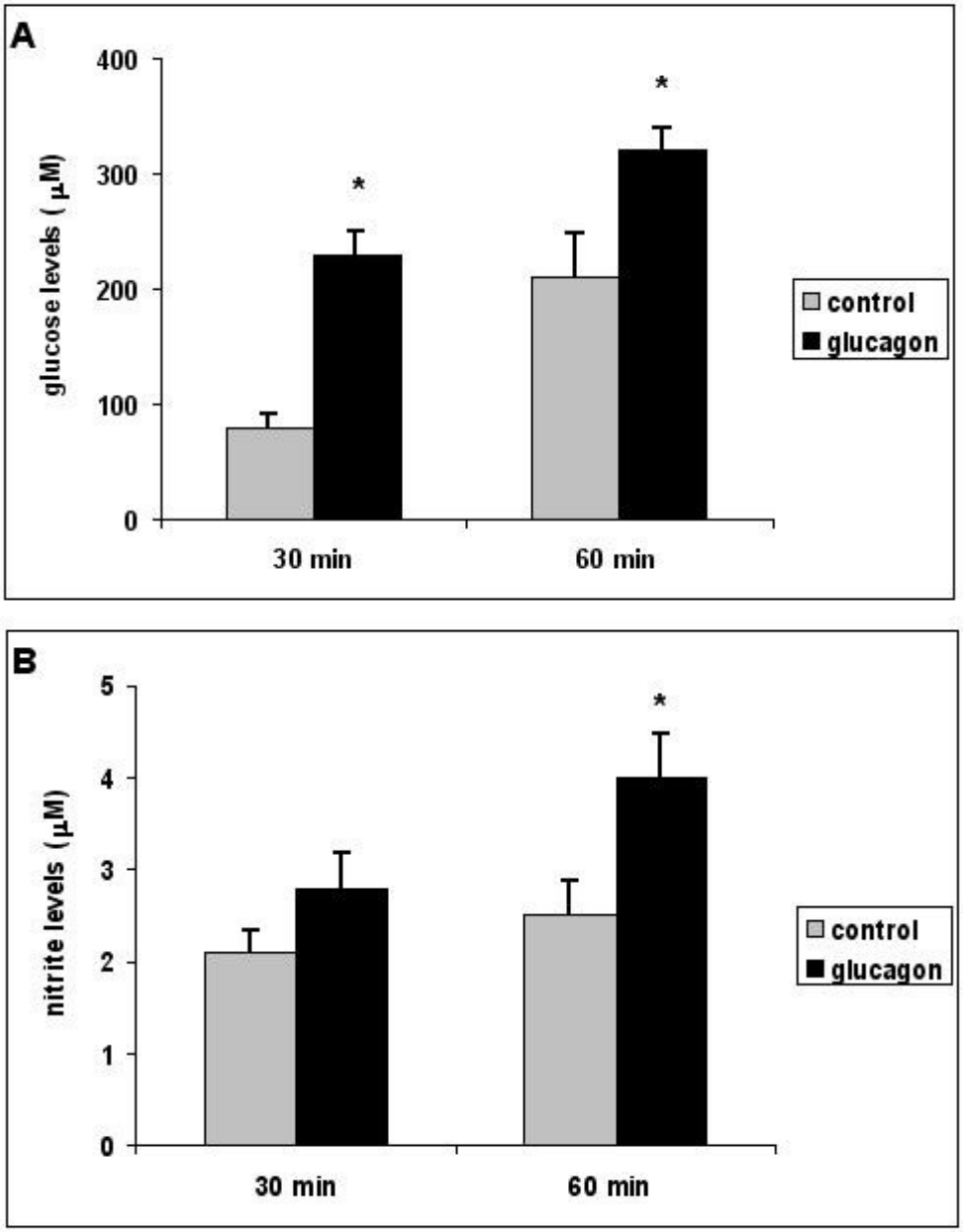
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Fig 2.

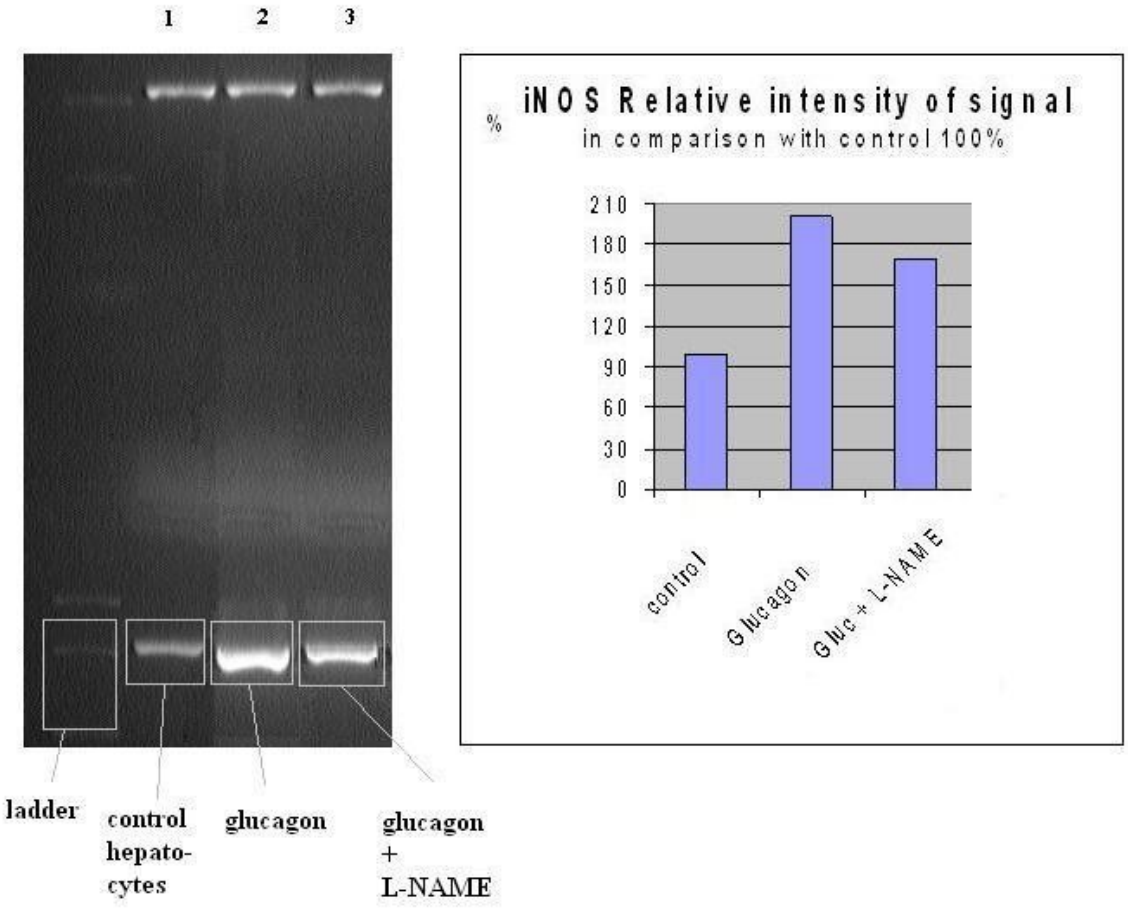
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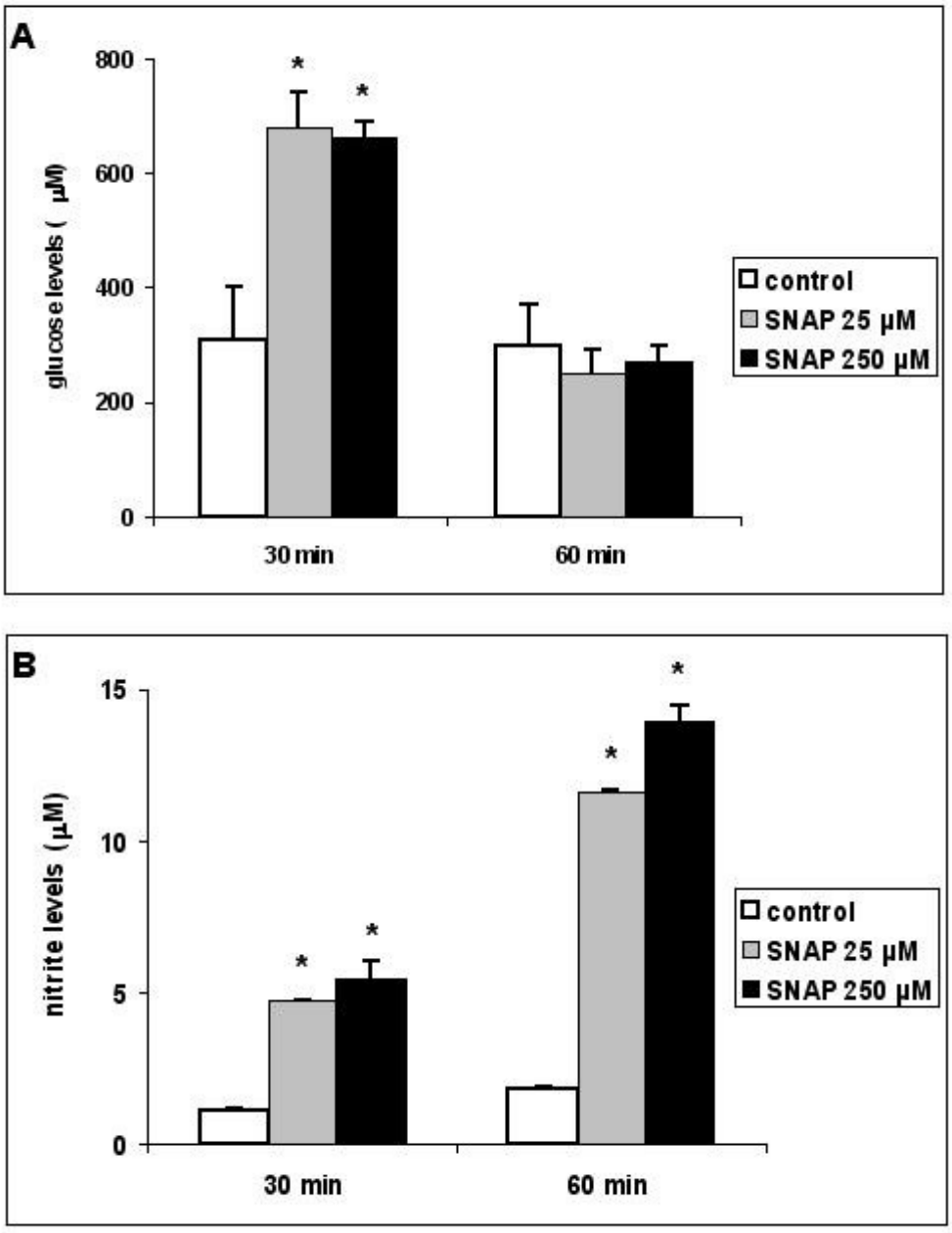
Fig 3.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