*Physiol. Res. 51: 387-394, 2002*

# Effect of Nitric Oxide Donors on Isoprenaline-Induced Lipolysis in Rat Epididymal Adipose Tissue: Studies in Isolated Adipose Tissues and Immobilized Perfused Adipocytes

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*Received October 10, 2001*
*Accepted January 10, 2002*

## Summary

The present investigation was directed to study the effect of *in vitro* or *ex vivo* NO donors, sodium nitroprusside and molsidomine, using isolated sliced adipose tissue or in the form of immobilized and perfused adipocytes on the basal and isoprenaline-stimulated lipolysis. The results demonstrated that 1) *in vitro* application of sodium nitroprusside to perfused adipocytes or molsidomine to sliced adipose tissues affects isoprenaline-induced lipolysis in two ways, an increase in lipolysis at low isoprenaline concentrations (which means the sensitization of adipose tissues to adrenergic effect by NO) and decreased adrenergic agonist-stimulated lipolysis at higher concentration of isoprenaline (a decrease in the maximum lipolytic effect of isoprenaline), 2) low concentrations of molsidomine alone induced lipolysis from adipose tissue which attained more than 60 % of that by isoprenaline ($pD_2$ value for molsidomine = 11.2, while $pD_2$ for isoprenaline = 8.17) while sodium nitroprusside did not affect the basal lipolysis significantly, 3) *in vivo* administration of molsidomine for 2 days reduced the maximum lipolytic effect of isoprenaline and (only non-significantly) increased the sensitivity to low doses of isoprenaline. In conclusion the present data demonstrate that NO plays an important role in adrenergic lipolysis in adipose tissues and further investigations are needed to unravel the exact role of NO in lipolysis.

**Key words**
Adipose tissue • Lipid mobilization • Nitric oxide • Isoprenaline • Molsidomine • Sodium nitroprusside

## Introduction

In adipocytes, which represent the largest reserve for lipids in the organism, both lipogenesis and lipolysis are taking place simultaneously. The mobilization of lipids from adipose tissue, hydrolysis of triglycerides to free fatty acids and glycerol is catalyzed by the hormone-sensitive enzyme lipase. The degree of lipolysis is a function of enzyme activation; for instance, this enzyme is stimulated by the growth hormone, and the sympathetic nervous system. The last stimulatory effect is mediated by $\beta_1$, and $\beta_3$ receptors, which are linked to the $G_s$ protein (Hirsch *et al.* 1989). The enzyme is inhibited by insulin, adenosine and also by the sympathetic nervous system; the latter is mediated by $\alpha_2$ receptors, which are linked to the $G_i$ protein (Arner 1992). The *in*



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*vivo* lipolysis in man is also affected by the parasympathetic nervous system (Anderson and Arner 1995), where some postganglionic sympathetic nervous fibers are cholinergic (Lindh and Hokfelt 1990).

As for lipolysis, lipogenesis in the adipose tissue is affected by nutritional and hormonal factors and is regulated mainly by lipoprotein lipase activity. Insulin increases the activity of lipoprotein lipase (Angel and Bray 1979) and supports lipogenesis in the adipose tissue. Catecholamines and growth hormone decrease lipoprotein lipase activity. A disturbance in the equilibrium between lipogenesis and lipolysis results in hypertrophy and hyperplasia of adipocytes with an increase or decrease in adipose tissue mass.

Circulating fatty acids, chylomicrons and very-low-density lipoproteins are the source of triglycerides in adipose tissues. Triglycerides can also be synthesized *de novo* in adipose tissues from precursors (e.g. glucose or lactate). The most important site for fatty acid synthesis is the liver. The adipose tissue is not only an important site of fatty acid and triglyceride synthesis but also of cholesterol synthesis, but to lesser extent than the liver (Angel and Bray 1979). Emphasis has been laid on lipid metabolism due to its importance to obesity and the contribution to cardiovascular pathophysiology.

Beside other well-known endogenous mediators, the role of endogenous nitric oxide (NO) in lipid metabolism in adipose tissue has recently been suggested. The significance of this endogenous free radical in a number of biological functions such as neurotransmission, cardiovascular, immune system, inflammation and many other physiological processes and pathophysiological situations has come under intensive investigation. NO is produced in mammalian cells through urea/NO pathway by the effect of an isoform of nitric oxide synthase (NOS) on its substrate L-arginine. At present, three isoforms of NOS are being cloned and identified, namely the neuronal NOS (nNOS, NOS I), endothelial NOS (eNOS, NOS III) and an inducible form (iNOS, NOS II). NOS I and III are constitutive and calcium-dependent while NOS II requires an induction signals. The expression of NOS II and NOS III was demonstrated in rat white adipose tissue (Ribiere *et al.* 1996) and more recently inducible nitric oxide synthase (iNOS) was demonstrated in human adipose tissue (Anderson *et al.* 1999).

Nevertheless, the role of NO in adipose tissue is ambiguous. The present work was directed to shed light on the role of NO on lipid mobilization from adipose tissues. The effect of NO donors on the basal and on isoprenaline-stimulated lipolysis in rat adipose tissue under *in vitro* conditions was quantitatively examined. The effect of NO donors on lipolysis was also studied in *ex vivo* experiments.

## Methods

*Materials*

Bovine serum albumin from Sigma (St. Louis, MO), molsidomine Sigma; Sea Plaque agarose (low-temperature gelling agarose, FMC, Rockland, ME), collagenase (Sevac, Prague) and diagnostic kits for lipid measurement (Lachema, Brno). All other chemicals were of analytical grade and were obtained from standard sources. Drugs were dissolved in a saline solution before application.

*Sliced adipose tissue and lipolysis evaluation*

Male Wistar rats (body weight 180–220 g) were fasted for 24 hours. Rats were decapitated and the epididymal fat pads were removed and kept in saline at 37 $^{o}$C. After drying on a piece of gauze, the fat pads were cut into small pieces and samples (50 mg) were incubated for 90 min in 1 ml Krebs-Ringer phosphate buffer (pH=7.4) containing 5 % bovine serum albumin. Drugs were added directly to the incubation medium; sodium nitroprusside (SNP), molsidomine and isoprenaline (ISO) in concentrations depicted in the figures were present in the incubation medium for the whole incubation time (90 min). The rate of lipolysis was determined by glycerol concentrations in the incubation medium according to earlier reports (Van Handel and Zilversmit 1957, Butler *et al.* 1961).

*Preparation of isolated adipocytes*

Epididymal fat pads from the male Wistar rats (body weight 180-220 g) were used for adipocyte isolation according to the modified method of Rodbell (1964). Adipose tissue was digested in a plastic vial with Krebs-Ringer bicarbonate buffer (KRB, pH=7.4) containing 3 % bovine serum albumin and 2 mg/5 ml/g of adipose tissue of collagenase. The adipose tissue was subjected to constant shaking at 37 $^{o}$C for 30 min. After this incubation period, fat cells were separated from connective tissue by filtration through a nylon mesh. The isolated cells were washed 4 times with glucose-free KRB that contained 1 % of bovine serum albumin. In the experiments, the number of adipocytes in 1 ml was counted several times and was found to be approximately 300 000 cells/ml.

*Immobilization and perfusion of isolated adipocytes*

For immobilization of the adipocytes we used the method described earlier for hepatocytes (Farghali *et al.* 1994, Farghali and Mašek 1998) with minor modification. The agarose solution kept in 37 °C was mixed 1:1 with the suspension of fat cells (usually 5 ml). The mixture was immobilized on agarose threads by extruding the agarose cell mixture through Chemfluor teflon TFE tubing (0.5 mm internal diameter) into medium in bioreactor tubes. The thin gel-adipocyte mixture through the thin tube was cooled to 18-20 °C instead of the original method of cooling to 4 °C (Lincová *et al.* 1994), where excessive cooling increased the rigidity of the phospholipid cellular membrane and consequently affected membrane functions (Lech and Calvert 1966, Vaughan and Steinberg 1965).

In these experiments, immobilized adipocytes were perfused in a recirculating manner. The perfusion rate was maintained constant throughout this time (8 ml/min). Single doses of ISO were added to the perfusion medium (in a volume of 1.0 ml) and cumulative lipolysis was assessed for 200 min. Then the immobilized adipocytes were washed out (3 times) and after stabilization, SNP in a concentration of $2x10^{-4}$ M was added (in a volume of 1.0 ml). 40 min later, the cumulative dose–response curve of ISO was repeated in the presence of SNP.

In all *in vitro* experiments, the lipolytic effect of the drug was assessed quantitatively. This means that the dose-response curve of the drugs was followed and the intrinsic activity (maximal lipolytic effect), and the affinity ($pD_2$ value – negative logarithm of the drug concentration that produces 50 % of the maximal effects) were estimated (van Rossum 1963).

*In vivo drug treatment and ex vivo experiments*

The rats which had fasted for 24 h received an intraperitoneal injection of SNP (0.1 mg/kg b.w. as 0.1 ml/100 g b.w.) and control rats were given an equivalent volume of saline solution. Four hours after the injection of SNP, the rats were killed by decapitation, blood samples were withdrawn and the serum lipid profile was estimated. Immediately after decapitation, the epididymal adipose tissue was removed and after slicing was used for *ex vivo* experiments for estimation of lipid mobilization after isoprenaline. By a similar procedure, two doses of molsidomine (0.2 mg/kg/day i.p. as 0.1 ml/100 g b.w.) were administered to rats on two successive days. The rats were killed 24 h after the second dose of molsidomine and were followed up according to the same procedure.

Plasma levels of total lipids, triglycerides and total cholesterol were estimated using commercial kits Lachema. Each experiment was repeated at least 6 times. Student's t-test was used for examining statistical significance.

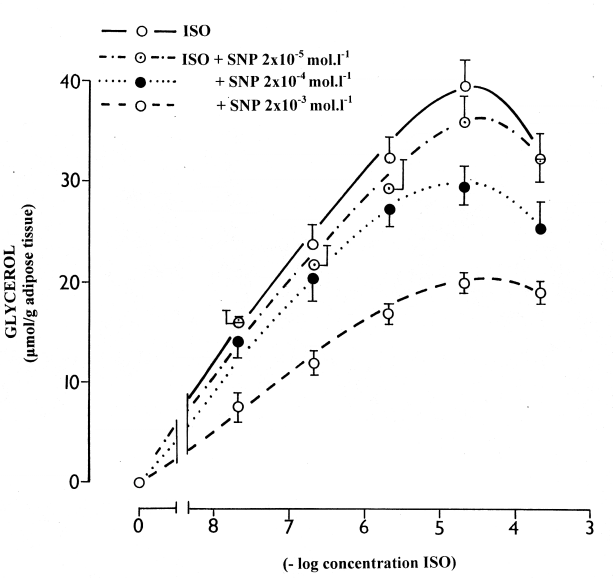


**Fig. 1.** *Effect of sodium nitroprusside (SNP) in concentrations of $2x10^{-6}$, $2x10^{-5}$ and $2x10^{-4}$ M on isoprenaline (ISO)-induced lipolysis in sliced adipose tissue. Abscissa: negative log of ISO molar concentration employed. Ordinate: the lipid mobilizing effect in µmol/g of adipose tissue. Mean values ± S.E.M.*

## Results

*The effect of NO donors on lipolysis in adipose tissues*

Figure 1 shows the effect of SNP pretreatment on isoprenaline-induced lipolysis in sliced adipose tissues presented as a conventional dose-response curve (van Rossum 1963). The relation between SNP doses ($2x10^{-5}$, $2x10^{-4}$ and $2x10^{-3}$ M) and the degree of ISO-induced lipolysis is presented.

SNP reduced ISO-induced lipolysis in a dose-dependant manner noncompetitively and the ISO-induced maximum lipolysis was reduced. At SNP concentration $2x10^{-5}$ M the reduction in ISO induced maximum lipolysis was non significant, at SNP concentrations $2x10^{-4}$ M, the lipolytic effect of ISO (at concentrations $2x10^{-6}$ – $2x10^{-4}$ M) was significantly ($p<0.05$ – $p<0.01$) reduced and at SNP concentration $2x10^{-3}$ M lipolysis was highly reduced ($p<0.001$) at all concentrations of ISO and

the maximum ISO-induced lipolysis was reduced to more than 60 %. SNP under the present experimental conditions did not affect the basal lipolysis in sliced tissues (data not shown).

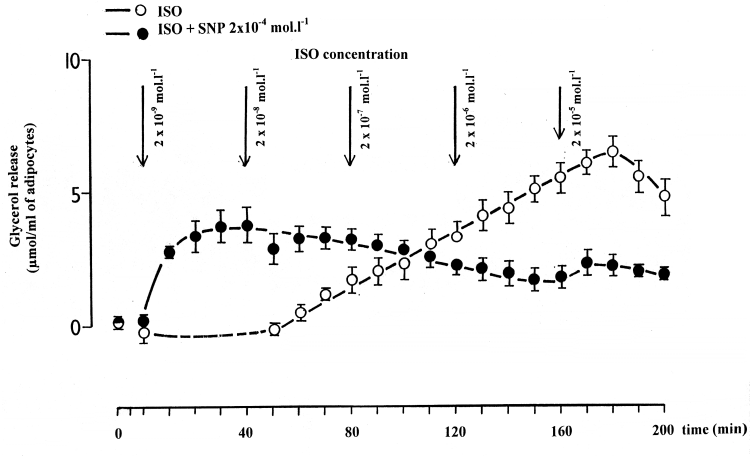


**Fig. 2.** *The time course of the lipid mobilizing effect of isoprenaline (ISO – white circles) in immobilized and perfused adipocytes and the effect of sodium nitroprusside (SNP) in concentrations of 2 x10$^{-4}$ M on isoprenaline-induced lipolysis (black circles) in immobilized and perfused adipocytes. Abscissa: time in min. Ordinate: glycerol release in μmol/ml of adipocytes. Mean values ± S.E.M.*

Figure 2 demonstrates the time course of the effect of SNP (2x10$^{-4}$ M) on basal and isoprenaline-induced lipolysis in immobilized perfused adipocytes. Under these conditions, SNP apparently produced a two-phase effect. As far as sliced tissues are concerned, SNP generally decreased the maximum of ISO-induced lipolysis ($p<0.01$ – $p<0.001$). However, SNP significantly ($p<0.01$ – $p<0.001$) increased the lipolytic effect of low concentrations of ISO (2x10$^{-9}$ – 2x10$^{-8}$ M), i.e. SNP increased the sensitivity of adipocytes to low concentrations of ISO.

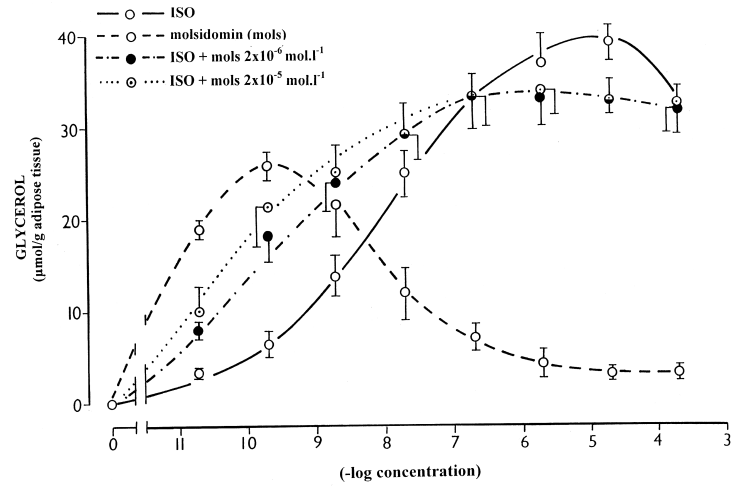


**Fig. 3.** *Lipid mobilizing effect of isoprenaline (ISO – continuous line) and molsidomine (broken line) in sliced adipose tissue. The influence of molsidomine in concentrations of 2x10$^{-6}$ and 2x10$^{-5}$ M on the dose response curve of ISO; mean values ± S.E.M.*

The effect of another NO donor, molsidomine, which releases NO without the mediation of the SH group (this may be the reason why tolerance to molsidomine is low) was followed in further experiments. Figure 3 demonstrates the effect of molsidomine on basal and ISO-induced lipolysis in sliced adipose tissues. The effect of molsidomine application alone, ISO alone or of ISO + molsidomine (in concentrations of 2x10$^{-6}$ and 2x10$^{-5}$ M) on lipolysis in adipose tissues was observed.

Molsidomine alone induced lipolysis from adipose tissue. The latter effect began at very low concentrations, peaked at 2x10$^{-10}$ M and corresponded to 67.4 % of ISO-induced maximum lipolysis. Estimates of pD2 were 11.2 and 8.17 for molsidomine and ISO, respectively. This means that the sensitivity of adipose tissue for molsidomine is by three orders higher than that for ISO.

At concentrations which did not affect basal lipolysis (2x10$^{-6}$ and 2x10$^{-5}$ M), molsidomine at both concentrations significantly increased ISO-induced lipolysis, specifically the effect of low concentrations of ISO in a dose-dependent manner (the dose-response

curve of ISO is shifted to the left, towards lower ISO concentrations). Estimates of $pD_2$ were 8.17 for ISO, 9.7 in the presence of molsidomine in the concentration of $2x10^{-5}$ and 10.1 for molsidomine in the concentration of $2x10^{-4}$ M.

Concomitantly, when ISO and molsidomine (in concentrations of $2x10^{-5}$ and $2x10^{-4}$ M) were administered simultaneously, the maximum lipolytic effect of high concentrations of ISO was decreased. Maximal lipolysis was reduced by 13 % and this effect was significant at the ISO concentration of $2x10^{-5}$ M ($p<0.05$).

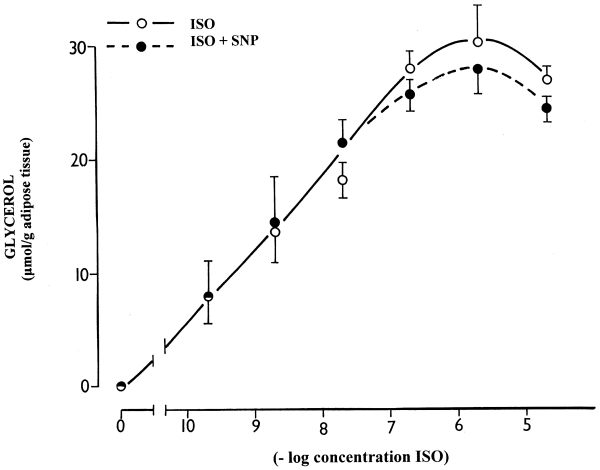


**Fig. 4.** *The effect of sodium nitroprusside (SNP) administered intraperitoneally in the dose 0.1 mg/ kg b.w. 4 h before killing the rat on the lipid mobilizing effect of isoprenaline (ISO) in sliced adipose tissue (ex vivo). Mean values ± S.E.M.*

The two-phase effect of NO on lipolysis in adipose tissues, which was evident with SNP in perfused adipocytes only, was evident with molsidomine even when using sliced adipose tissue.

The effect of *in vivo* administration of NO donors on adrenergic lipolysis in adipose tissue was followed. SNP was applied (0.1 mg/kg i.p.) 4 hours before sacrificing the rats and the effect on ISO-induced lipolysis (*ex vivo*) and plasma lipid parameters was observed.

Figure 4 demonstrates the effect of *in vivo* administration of SNP on ISO-induced lipolysis in adipose tissue. SNP *in vivo* did not reduce ISO-induced lipolysis significantly compared to *in vitro* data (which could be due to clearance of SNP from the tissue).

The pretreatment with SNP reduced total plasma lipids nonsignificantly and the triglyceride levels remained unchanged (data not shown).

The intraperitoneal administration of molsidomine for two successive days (0.2 mg/kg/day) (Fig. 5) demonstrated that molsidomine affected the maximum lipolytic action of ISO in adipose tissue (*ex vivo* experiment). Molsidomine significantly ($p<0.05$ and 0.001) reduced the maximum lipolytic activity of high ISO concentrations (ISO $2x10^{-6}$ and $2x10^{-5}$ M). In this connection, molsidomine pretreatment *in vivo* produced the same effect on adrenergic lipolysis in *ex vivo* adipose tissues as after *in vitro* application, but without an appreciable influence on the lipolytic effect of low ISO concentrations.

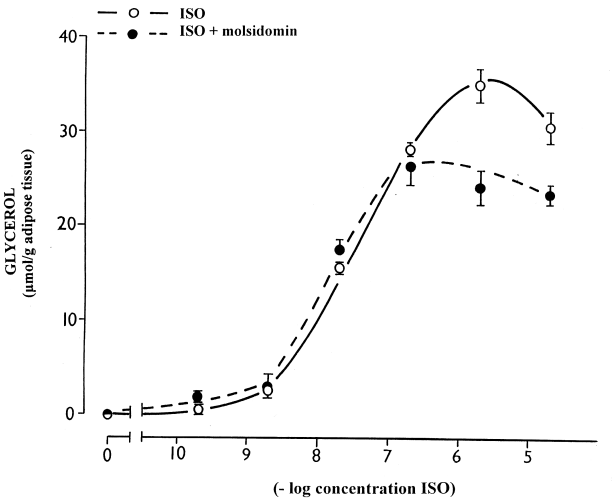


**Fig. 5.** *The effect of molsidomine administered for 2 successive days in doses of 0.2 mg.kg$^{-1}$b.w. intraperitoneally on lipid mobilizing effect of isoprenaline (ISO) in sliced adipose tissue ex vivo. Mean values ± S.E.M.*

## Discussion

In spite of the evidence that both constitutive and inducible NOS are expressed in the adipose tissue (Ribiere *et al.* 1996), the role of NO in this tissue still is ambiguous. Due to the physicochemical nature of NO, adipose tissues may significantly contribute to NO production in the whole organism, for example, during endotoxemia. Abnormality has been shown in NO biosynthesis in cardiovascular patients with hyperlipoproteinemia. Changes in NO activity may affect the metabolism of lipoproteins containing apo B (Kaye *et al.* 1998). Moreover, it has been indicated that NO donors or

precursors are able to decrease the diet-induced rise in LDL (Kurowska and Carrol 1998). Hypertriglyceridemia belongs to the major changes in lipid metabolism during infection or endotoxemia. The elevation in circulating triglyceride levels can be the result of an increase in hepatic secretion of very-low density lipoproteins and from alterations in the clearance rate of triglycerides. It has been shown (Picard *et al.* 2001) that bacterial lipopolysacharides (LPS) cause hypertriglyceridemia through a generalized decrease in the activity of lipoprotein lipase and stimulate the expression and activity of inducible NOS in the adipose tissue and skeletal muscles. As the dose of endotoxin increases, the contribution of hepatic triglyceride secretion to hypertriglyceridemia decreases and triglyceride clearance becomes more important. LPS-induced reduction in skeletal muscle lipoprotein lipase activity is posttranscriptionally mediated by iNOS-induced NO production. Moreover, the latter authors emphasize that the activity of adipose tissue lipoprotein lipase during endotoxemia is reduced by a mechanism other than iNOS-induced NO production and this mechanism does not involve changes in insulinemia.

Our *in vitro* data that deal with the effect of NO donors (SNP and molsidomine) on adipose tissue lipolysis suggest a possible dual role of NO on ISO-stimulated lipolysis. On the one hand, NO reduces the maximum of lipolysis stimulated by high levels of ISO and, on the other, it potentiates lipolysis at low ISO concentrations. This possible dual effect is dose dependent.

The NO dual effects were observed in other tissues as in the β cells of the pancreas where NO plays a role as a physiological mediator and is also involved in pathophysiological processes (Spinas 1999). Dual beneficial and deleterious effects of NO were observed in other tissues or organs such as the liver and others (Farghali *et al.* 1997).

Quantitative assessment of the effect of NO on dose-response curve of ISO-stimulated lipolysis revealed that both SNP and molsidomine inhibited ISO-stimulated lipolysis noncompetitively. This is illustrated in Figures 1-3 where the inhibition affected maximum lipolysis, but did not produce a parallel shift of the dose response curve to the right. Therefore, the interference between NO and ISO lipolytic action could not act at the level of β–adrenergic receptors. Moreover, the effect of molsidomine on ISO-stimulated lipolysis when used in *ex vivo* experiments was qualitatively similar to the *in vitro* results.

The well-known effects of NO are related to its activation of soluble guanylyl cyclase with the consequent increase in cGMP in cells. The first evidence for modulation of the lipolytic process in adipocytes by NO donors was given by Gaudiot *et al.* (1998). These authors found that nitrosothiol donors (e.g. SNAP) differ from other donors as PAPA-NONOate in that the former increased the basal release of glycerol from rat adipocytes and that the SNAP lipolytic effect is not related to guanylyl cyclase activation but to the nitrosylation of phosphodiesterases. Even differences between individual NO donors was found through their effect on stimulated lipolysis (Gaudiot *et al.* 1998). Nitrosothiols decreased catecholamine- but not forskolin- or cyclic dibutyryl AMP-stimulated lipolysis. On the other hand, PAPA-NONOate decreased the induced lipolysis regardless of the stimulus used. This indicated that the NO-related species may exert opposite effects in the control of lipolysis and it was suggested that NO modulates lipolysis through different mechanisms that appear to depend on the redox forms of NO but are not related to activation of guanylyl cyclase.

The role of endogenously produced NO by adipocytes in lipolysis regulation was also studied (Gaudiot *et al.* 2000) by using NOS inhibitor DPI (diphenyl iodonium). DPI inhibits NOS in a dose-dependent manner and is capable to suppress completely NO synthesis in adipocytes. This drug was found to decrease both basal and stimulus-induced lipolysis of various origin (ISO, cyclic dibutyryl AMP). The antilipolytic effect of DPI was prevented not only by NO donors SNAP, but also by antioxidants. The same antilipolytic effect as DPI was found after the exposure of adipocytes to an extracellular system generating oxygen species or $H_2O_2$. It was shown that as DPI as $H_2O_2$ decreases the activation of cAMP-dependent protein kinase (PKA). The antilipolytic effect of DPI is related to adipocyte NOS inhibition and this inhibition leads to PKA alterations.

In our experiments we have not shown the effect of SNP on basal lipolysis in adipose tissues. Molsidomine *per se* increased the release of glycerol from adipose tissues in a dose-dependent manner with maximum lipolytic effect reaching more than 60 % of that of ISO. An estimate of $pD_2$ (the negative logarithm of the concentration that produces 50 % of the maximal effect) for molsidomine was by three orders higher than that of ISO. Our unpublished data regarding the effect of *in vivo* repeated administration of molsidomine on lipid levels in

the plasma showed a significant reduction of triglycerides and total cholesterol.

Based on our data and other reports it is evident that NO production in adipose tissues plays a significant regulatory role in basal and stimulated lipolysis. Our own data provide evidence that exogenous NO applied to adipose tissues plays a significant modulatory effect on catecholamine-stimulated lipolysis. Moreover, the decreased ISO-stimulated lipolysis by NO is not produced competitively, i.e. at the level of β receptors. Further intensive studies are required to unravel the multiple effects of NO in lipolytic processes.

## Acknowledgements

This work was supported by the research grants from MZ NL/5885-3, GAČR 305/00/1129 and Project of Ministry of Education of the Czech Republic J13/98:111100002

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