Inconsistent Effect of Nitric Oxide on Lipolysis in Isolated Rat Adipocytes

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Summary

Though two isoforms of nitric oxide synthase, iNOS and eNOS, were reported in adipocytes, the role of NO in adipose tissue is still ambiguous. The aims of the present study were 1) to follow the effect of bacterial lipopolysaccharide (LPS), on 24 h-lipolysis in rat epididymal adipocyte culture in relation to iNOS stimulation; 2) to compare LPS-induced NO effects with exogenously NO, delivered as S-nitroso-N-acetylpenicillamine (SNAP), and 3) to examine the possible role of NO signaling agonist in lipolysis mediated by the β_3-adrenoreceptor agonist. Lipolysis was measured by glycerol and free fatty acid (FFA) production. The medium nitrite levels were used for the indirect estimation of NOS expression. Adipocyte mitochondrial function was assessed by the MTT test. LPS produced a concentration-dependent increase of NO with a decrease of viability at the highest dose. However, LPS did not affect lipolysis. SNAP did not exhibit significant changes in glycerol, FFA or MTT. BRL-37344 and db-cAMP significantly increased nitrite, glycerol and FFA levels. There was a positive correlation between glycerol release and nitrite production. Moreover, BRL-37344 significantly reduced mitochondrial functions. The pretreatment with bupranolol, β_3-antagonist, restored all parameters affected by BRL-37344. These results support a concept that NO fulfils multifaceted role of stimulating lipolysis under physiological conditions (β-agonistic effect) and modulating the same processes during inflammatory (LPS) processes.

Introduction

It is well established that adipocytes are the most important cells as determinants of energy homeostasis and metabolic complications of obesity. Therefore, more attention is needed for intensive research aimed at the mechanisms regulating adipose tissue metabolism and their potential pharmacological modulation. Adipocytes act as endocrine/secretory cells and not only as passive receptacles for the storage of excess energy in the form of fat. Several types of signals have been established to modulate adipose tissue functions (Hellerstein and Munro 1994, Spiegelman and Flier 1996, Ribiere *et al.* 1996, Andersson *et al.* 1999, Kapur *et al.* 2000, Frühbeck and Gómez-Ambrosi 2001, Coppack 2001).

Among the key signals that were found in practically all tissues including the adipose tissue is nitric oxide (NO). Several years ago, a role of endogenous NO in lipid metabolism in adipose tissue was suggested. It was reported that adipocyte endogenous production of

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NO is required for lipolytic activity of this cell and that the inhibition of nitric oxide synthase (NOS) modulate lipolysis (Gaudiot *et al.* 2000). The expression of isoforms of NOS, constitutive endothelial (eNOS) and/or inducible (iNOS), was demonstrated in rat white adipose tissue (Kapur *et al.* 2000, Giordano *et al.* 2002). Both enzymes were also demonstrated in human adipose tissue (Andersson *et al.* 1999). As in the case with several other cell types, an endotoxin challenge significantly increases cellular iNOS protein concentration and activity in both white and brown adipose tissues. It was reported that *in vivo* NO mediates endotoxin-induced hypertrigly-ceridemia through its action on skeletal muscle lipoprotein lipase (Picard *et al.* 2001).

Besides hormones, growth factors, cytokines and different types of receptors that are expressed in white adipose tissue, catecholamines possess a well-known lipolytic effect on fat cells through different subtypes of β-adrenoreceptors. The decreased function of β3-adreno-receptors could be associated with impairment of lipolysis in white fat or with thermogenesis in brown fat (Spiegelman and Flier 1996). Recently we have reported that β-adrenergic lipolysis, produced *via* β3-adrenoreceptor/adenylyl cyclase/cAMP/protein kinase A (PKA)-signaling cascade, involves NO production downstream of β3-adrenoreceptor/cAMP-pathway (Canová *et al.* accepted).

In spite of the involvement of NO in adipocyte metabolic signaling and the presence of NOS isoforms in adipocytes, the role of NO in adipose tissue is still ambiguous. The aim of the present study was 1) to follow up the effect of endotoxin that is induced by application of bacterial lipopolysaccharide (LPS), on lipolysis in rat epididymal adipocyte cultures in relation to stimulation of the expression of iNOS; 2) to elucidate the role of NO signaling in β-adrenoreceptor agonist-induced lipolysis; and 3) to compare the involvement of endogenously produced NO or exogenously delivered NO in lipolytic activity. To achieve these goals, rat epididymal adipose tissue was used for adipocyte isolation. NO production was assessed under the influence of LPS, β3-adrenergic receptor agonist and agonist/antagonist pair or under the influence of cyclic nucleotide cAMP in relation to lipolysis occurring in adipocyte cultures. S-nitroso-N-acetyl penicillamine (SNAP) was used as NO donor (exogenous source of NO) and its effect on lipolysis was compared to that of endogenously produced NO.

Material and Methods

Experimental animals

Male Wistar rats, weighing 250-300 g, were purchased from Velaz (Lysolaje, Czech Republic) and housed in separated cages in a temperature- and light-controlled room (12-h dark/light cycle). They received adequate care and were provided with water and standard laboratory chow *ad libitum*. All procedures were performed in accordance with the general guidelines of the First Faculty of Medicine, Charles University in Prague.

Chemicals and reagents

Dulbecco′s Modified Eagle′s Medium high glucose (DMEM), William′s medium E without phenol red, penicillin-streptomycin solution 100x, L-glutamine 200 mM, bovine serum albumin fraction V (BSA), dibutyryl cyclic-AMP sodium-potassium salt (db-cAMP), S-nitroso-N-acetyl penicillamine (SNAP), (([+/-]-[R,R]-[4-(2-[3-chlorophenyl]-2-hydroxyethyl)amino]propyl) phenoxy] acetic acid) sodium salt (BRL-37344); MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), sulfanilic acid, N-(1-naphtyl)ethylendiamine dihydrochloride and bicinchoninic acid protein assay kit (BCA) were obtained from Sigma-Aldrich (Prague). Bupranolol (1-(2-chloro-5-methylphenoxy-3-[(1,1-dimethylethyl)amino]-2-propanol hydrochloride) was a gift from Schwarz Pharma (Zwickau, Germany). Gibco fetal bovine serum (FBS) was purchased from KRD (Prague). Crude collagenase was obtained from Sevapharma (Prague). Nitrite/Nitrate calorimetric method modified for 96-well plates were bought from Roche Diagnostic (Prague) and Randox Kit for nonesterified free fatty acids from Randox (Břeclav, Czech Republic). All other chemicals were obtained from standard sources and wereof the highest grade available.

Adipocyte isolation

Rats were fasted for 24 h before the experiment and then they were killed by cervical dislocation. The epididymal fat pads were removed and kept in saline at 37 ^{0}C. Dissected epididymal fat pads were pooled for adipocyte isolation by collagenase digestion as previously mentioned (Rodbell 1964, Lincová *et al.* 2002). Briefly, the adipose tissue was digested with Krebs-Ringer bicarbonate buffer (KRB, pH =7.4) containing 2 % BSA and 2 mg collagenase/5 ml/2 g of adipose tissue and subjected to constant shaking at 37 ^{0}C for 60 min.

Adipocytes were separated by filtration through a nylon mesh (150 μm). The isolated cells were washed 4 times with glucose free KRB that contained BSA.

Primary rat adipocyte culture and treatments

Isolated adipocytes were incubated under sterile conditions in a DMEM medium supplemented with 2 mM glutamine, 10 % FBS, 100 μg/ml streptomycin and 100 IU/ml penicillin. 150 μl of packed adipocytes were placed in 750 μl of enriched DMEM in sterile polystyrene tubes loosely covered by lids and maintained at 37 ^{0}C, 95 % air and 5 % CO_2. After 2-h stabilization, a second 750 μl of supplemented DMEM were added and cells in the suspension were treated with either bacterial lipopolysaccharide (LPS; 5, 10, 15 μg/ml), NO donor (SNAP; 50, 100 and 200 μM), specific $β_3$-agonist (BRL-37344; 2×10^{-7} M) or dibutyryl cAMP (db-cAMP, 1 mM). In some experiments, LPS (10 μg/ml), SNAP (200 μM) or $β_3$-specific competitive antagonist (bupranolol; 1×10^{-5} M) pretreatments were used before BRL-37344, which was applied after 30 min directly to the culture medium in a volume of 15 μl to obtain the appropriate final concentration. The treated and untreated adipocytes were incubated for 24 h without shaking. After that, 1400 μl of infranatant were removed for further measurements and floating adipocytes were harvested. They were processed for the MTT test or frozen at –80 °C for total protein assessment.

Nitric oxide production assays

NO production was determined by measuring its oxidation products, NO^{2-} and NO^{3-}, which were detected colorimetrically (540 nm) in individual cell-free media incubated for 10 min at room temperature with an aliquot of Griess reagent (1 % sulfanilamide, 0.1 % naphtyl-ethylendiamine, 2.5 % H_3PO_4) and/or by using Roche nitrite/nitrate assay kit. The nitrite levels were calculated from a $NaNO_2$ standard curve.

Evaluation of lipolysis

Glycerol medium levels were determined spectrophotometricaly as described previously (Lambert and Neish 1950). Medium free fatty acid (FFA) levels were measured and calculated as concentration of FFA according to Randox kit instructions.

MTT test

MTT test as a mitochondrial function assay was used to evaluate the cell reduction of MTT to insoluble purple formazan by mitochondrial dehydrogenase which is produced by viable cells. The MTT test was performed according to manufacturer's instruction with slight modifications. Briefly, at the end of experiments the adipocytes were cultivated for 3 h in MTT work solution, consisting of 500 μg/ml MTT and serum-free William's medium E. Then the MTT work solution was removed and the formazan was solubilized using DMSO. The resulting purple solution was measured spectrophoto-metrically (540 nm) yielding absorbance as a function of concentration of the converted dye. There was an increase of linearity between MTT-derived formazan and cell density.
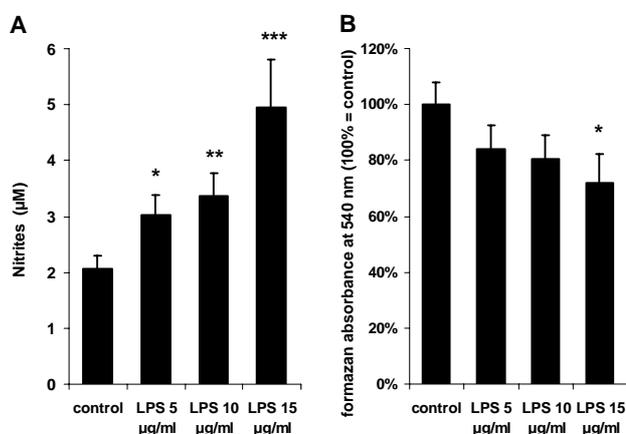
Fig. 1. The effect of lipopolysaccharide (LPS) on nitrite production **(A)** and MTT reduction **(B)** in adipocytes. Isolated adipocytes were cultivated with LPS in increasing concentrations of 5, 10 and 15 μg/l. After 24 h, the medium levels of NO_2^- as the end product of NO production were estimated (A) and adipocytes were further processed to detect reduction of MTT to an insoluble purple formazan by mitochondrial dehydrogenase in viable cells (B). Data are expressed as mean values ± S.E.M. Student's t-test for unpaired data: * p<0.05, ** p<0.01, *** p<0.001 vs. control.

Determination of adipocyte total protein

The total cell protein content from lysate of isolated adipocytes was assessed by Sigma BCA assay kit. In most of the experiments, the protein content of adipocytes was found to be practically constant with an average of 7.9±0.3 mg protein/ml of packed adipocytes. Therefore, the results were recalculated per 100 μl of the packed adipocytes.

Statistical analysis

All results were expressed as the means ± S.E.M. of separate experiments (means of at least of 9 values). The statistical significance of the mean difference was determined using Student's t-test for unpaired data or one-way ANOVA with *post hoc* Turkey-

Kramer multiple comparison test. P<0.05 values were considered to be statistically significant.
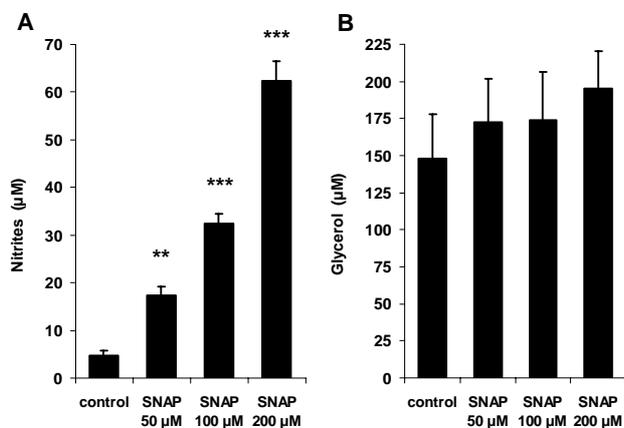
Fig. 2. The effect of S-nitroso-N-acetyl penicillamine (SNAP; 50, 100 and 200 μM) on nitrite production **(A)** and lipolysis **(B)** by adipocytes. After 24 h, the medium levels of NO_2^- (A) and glycerol release (B) were estimated. Data are expressed as mean values ± S.E.M. Student's t-test for unpaired data: ** p<0.01, *** p<0.001 vs. control.

Results

Effects of LPS on nitric oxide production, lipolysis and MTT reduction

LPS produced a concentration-dependent increase in NO production as demonstrated in Figure 1A. Lipolysis after 24 h of incubation of LPS with adipocyte was variable, being unaffected in most experiments. Moreover, the LPS effects on BRL-37344-induced lipolysis were not significant (data not shown). LPS at the highest concentration (15 μg/ml) significantly inhibited mitochondrial function as revealed from the MTT test and is depicted in Figure 1B.

Effects of SNAP on nitric oxide production, lipolysis and MTT reduction

Figure 2 shows that the exogenous NO donor, SNAP, giving nitrite levels as high as 60 μM from 200 μM SNAP addition (Fig. 2A) did not modify significantly either glycerol (Fig. 2B) or FFA levels or the MTT test (data not shown) in 24-h adipocyte cultures. Similarly to LPS, SNAP pretreatment did not influence BRL 37344-induced lipolysis.

Effects of BRL-37344 and bupranolol + BRL-37344 on nitric oxide production, lipolysis and MTT reduction

BRL-37344 significantly increased both glycerol (Fig. 3A) and FFA (Fig. 3B) levels. Nitrite levels were

enhanced by BRL-37344 (Fig. 4A) in the same pattern as depicted in Figure 3B for glycerol. It is clear that bupranolol pretreatment reduced both BRL-induced lipolysis and nitrite generation in adipocytes. BRL-37344 significantly reduced formazan formation from MTT that was restored to control values by bupranolol (Fig. 4B).
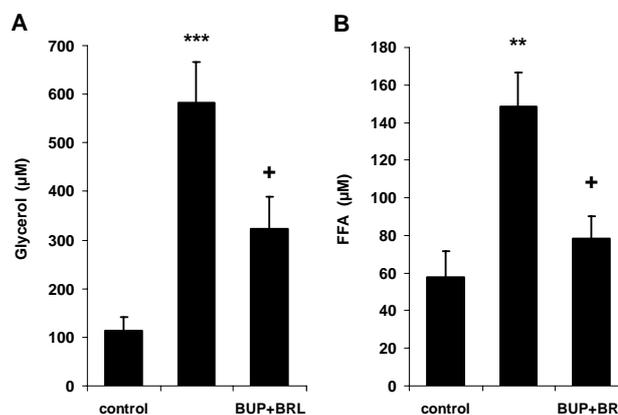
Fig. 3. The effect of BRL-37344 and bupranolol+BRL-37344 pair on lipolysis in adipocytes. Isolated adipocytes were cultivated for 24 h with single concentration of BRL-37344 (BRL 0.2 μM) and bupranolol (BUP 10 μM) added to the medium 30 min before BRL (BUP+BRL). At the end of incubation period, the medium levels of glycerol **(A)** and fatty free acids, FFA, **(B)** were estimated. Data are expressed as mean values ± S.E.M. One-way ANOVA with *post hoc* Turkey-Kramer multiple comparison test: ** p<0.01 and *** p<0.001 vs. control, + p<0.05 vs. BRL.
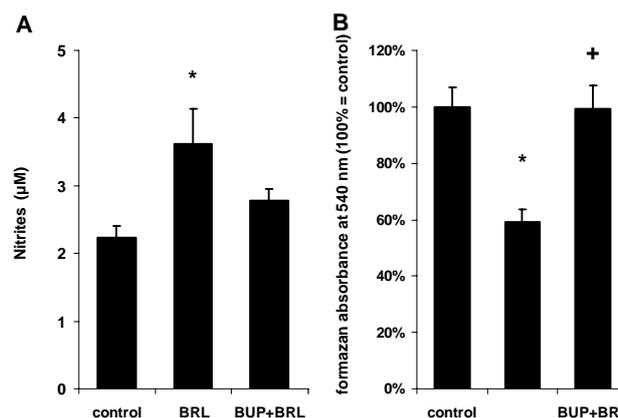
Fig. 4. The effect of BRL-37344 and bupranolol+BRL-37344 pair of nitric oxide production and MTT. Isolated adipocytes were cultivated with single concentration of BRL 37344 (BRL 0.2 μM) and bupranolol (BUP 10 μM) added to the medium 30 min before BRL (BUP+BRL). After 24 h, the medium levels of NO_2^- (A) and reduction of MTT to an insoluble purple formazan by mitochondrial dehydrogenase of viable cells (B) were estimated. Data are expressed as mean values ± S.E.M. One-way ANOVA with *post hoc* Turkey-Kramer multiple comparison test: * p<0.05 vs. control, + p<0.05 vs. BRL.

Effects of db-cAMP on nitric oxide production and lipolysis and correlation analysis

Figure 5A demonstrates that db-cAMP significantly increased both glycerol and nitrite levels. There was highly positive correlation (p<0.001) with r^2 being 0.70 between glycerol release and nitrite production for pooled data as obtained after BRL-37344, BRL-37344/bupranolol pair or db-cAMP experiments (Fig. 5 B).

Discussion

Currently the role of nitric oxide in lipolysis is being investigated both *in vitro* and *in vivo*. In the present study, we have addressed the potential role of endogenously produced NO (including both LPS-induced iNOS isoform and constitutive eNOS isoform stimulated by β₃-adrenoreceptor agonist) or exogenously added NO (delivered from SNAP) on lipolytic events in rat adipocyte culture. NO generated under various treatments produced different profiles of lipolysis in adipocyte culture. It was reported that in sepsis and endotoxemia (i.e. *in vivo*), few hours after LPS injection, metabolism is characterized by catabolism which resulted in active lipolysis in adipose tissues to a various extent depending on whether they are of subcutaneous or mesenteric origin (Sugawara *et al.* 2003). Our data show that a lipolytic response of cultured white rat adipocytes to LPS was not evident in spite of the increased NO production at various LPS concentrations. However, when sliced rat epididymal adipose tissues were exposed to LPS for a few hours, lipolysis was increased (unpublished data) which confirm other report (Sugawara *et al.* 2003). Kapur *et al.* (2000) suggested that NO may be implicated in the regulation of metabolism in adipose tissue and skeletal muscle. These authors uncovered the role of NO in modulation of glucose transport and lipoprotein hydrolysis. It is reasonable to dissociate between LPS/NO effects *in vivo* and *in vitro* with regard to the adipose tissue and isolated adipocytes. Adipocytes can synthesize and secrete several cytokines, complement system products and the macrophage colony-stimulating factor. These factors are important modulators, stimulators and/or suppressors of leptin secretion, β₃-adrenoreceptor expression and other effects (Coppack 2001, Schling and Löffler 2002). The paracrine and endocrine regulators derived from adipose tissues, due to the highly complex nature of *in vivo* conditions, are expected not to be identical with the *in vitro* situation at least quantitatively

if not qualitatively. Moreover, NO released from SNAP failed to stimulate lipolysis in adipocyte cultures. On the other hand, β₃-adrenoreceptor agonist BRL-37344 and db-cAMP, a stable congener to cAMP, increased NO production from rat adipocytes. This *in vitro* study demonstrates that NO is involved in cAMP-dependent lipolysis. The selective β₃-blocker bupranolol inhibited not only BRL-37344-induced lipolysis but also NO production. Under these pharmacological manipulations, there was a positive correlation between nitrite levels and the degree of lipolysis as measured by glycerol formation. Indeed, the present data demonstrate that at least a part of β-adrenergic lipolysis occurs *via* the β₃-adrenoreceptor/ adenylyl cyclase/protein kinase A cascade and cAMP-signaling pathway and that NO produced downstream of β₃-adrenoreceptor/cAMP is involved in lipolysis. The role of β₂- or β₃-adrenoreceptor/cAMP stimulation of various kinases that mediate glucose uptake in brown adipocytes and other tissues was reported (Chernogubova *et al.* 2004). We can hypothesize that some of these kinases can be responsible for stimulation of eNOS and/or iNOS enzyme activity. Moreover, it was reported that cAMP regulates iNOS expression in 3T3-L1 cell-derived adipocytes through modulating nuclear factor-κB (NF-κB) activity, which is responsible for iNOS gene transcription (Dobashi *et al.* 2003).
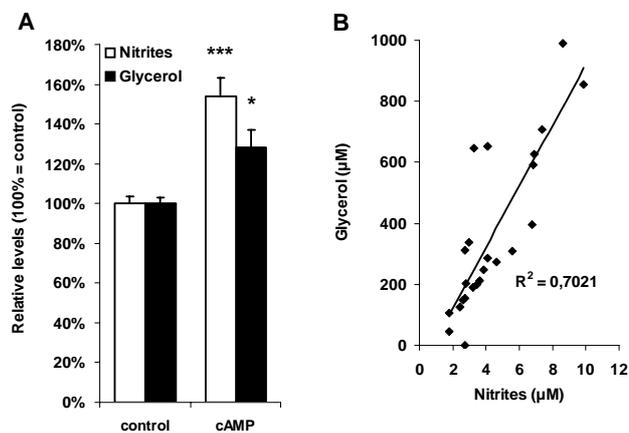
Fig. 5. Effect of db-cAMP (1 mM), on nitric oxide production and lipolysis **(A)** and the correlation between the medium nitrite and glycerol levels for pooled data as obtained after db-cAMP (1 mM), BRL-37344 (BRL 0.2 μM) and bupranolol 10 μM /BRL-37344 pair (BUP+BRL) experiments **(B)**. After 24 h adipocyte cultivation, the medium levels of NO₂⁻ as the end product of NO production and glycerol release as a marker of lipolysis were estimated. The nitrite and glycerol concentrations (**A**) were expressed as relative mean values ± S.E.M. Student's t-test for unpaired data: *** p<0.001 vs. control for nitrite, * p<0.05 vs. control for glycerol. The highly positive correlation (**B**) between glycerol release and nitrite production in the medium is illustrated.

The well-known diversified and multifunctional role of NO in biological systems depends on its levels, type of tissue, location, redox status and whether the study is carried out *in vivo* or *in vitro* (Farghali *et al.* 2003). In the present study, the NO donor SNAP, added at various concentrations to rat white adipocyte cultures failed to significantly alter lipolysis. It was reported that high concentrations of NO can inhibit lipolysis stimulated by different pathways, but low levels of intracellular NO, on the other hand, are necessary for both basal and stimulated lipolysis, since they maintain protein kinase A in an active state due to antioxidant properties of NO (Gaudiot *et al.* 2000).

Using adipocyte cultures, it seems that the decrease in MTT reduction due to β$_3$-adrenoreceptor agonist/NO reflects reversible decrease in mitochondrial function due to the lipolytic pathway rather than a real toxic (irreversible) consequence of NO release. This is supported by our data, which show that high concentration of SNAP-released NO (about tenfold more compared to β$_3$-adrenoreceptor agonist-induced NO release) neither affected lipolysis nor the MTT reduction test. On the other hand, the highest dose of LPS decreased adipocyte viability as assessed by the MTT test most likely by its own toxic effect.

In conclusion, the use of adipocyte cultures better defines the major cellular structures responsible for body lipid homeostasis and that NO belongs to the important signals that regulate this process under physiological and certain pathological conditions related to inflammation. The inconsistence of the effects of NO on lipolysis in rat adipocytes supports the concept that NO fulfils multifaceted signal of stimulating lipolysis under physiological conditions (e.g. β-adrenoreceptor agonistic effect) and modulating the same process under inflammatory (e.g. the LPS effect) processes. Finally, results concerning NO lipolytic involvement in adipocyte cultures cannot be simply extrapolated to *in vivo* conditions.

Acknowledgements

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