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Modification of Adipocyte Membrane Adenylyl Cyclase Activity by NAD: Evidence against NAD-Induced Endogenous ADP-Ribosylation of Gsα Protein

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Treatment of saponin-permeabilized adipocytes with NAD enhanced adenylyl cyclase activity stimulated by GTγS, $[Al/F_4]^-$, isoproterenol, and forskolin in membrane fractions and potentiated isoproterenol-induced cAMP accumulation in whole cells. In parallel, when permeabilized adipocytes were incubated with [^{32}P]NAD, there was significant incorporation of [^{32}P]ADP-ribose in a 44-kDa acceptor membrane protein. This reaction was inhibited by L-arginine and was enhanced by the addition of GTPγS. Surprisingly, this 44-kDa protein could not be identified as Gs protein: (1) It was not recognized by Gsα specific antibody; (2) it did not comigrate with the major cholera toxin substrates in either 10% SDS-PAGE or two-dimensional electrophoresis; (3) a pretreatment of adipocytes with NAD did not decrease cholera toxin-mediated ADP-ribosylation of Gsα proteins on membrane fractions. Our results indicate that NAD did not induce endogenous ADP-ribosylation of Gsα in permeabilized rat adipocytes but nonetheless modified the adenylyl cyclase response.

Mono-ADP-ribosylation is a covalent modification in which the ADP-ribose moiety of NAD is transferred to specific amino acid residues in acceptor proteins (1). The enzymes which catalyze this reaction, the mono-ADP-ribosyltransferases represent a large family of enzymes found in eukaryotes, viruses, plants and bacteria (2). The significance of mono-ADP-ribosylation has been well documented for bacterial toxins such as cholera toxin and pertussis toxin. Cholera toxin ADP-ribosylates an arginine residue of Gs (1), whereas pertussis toxin ADP-ribosylates a cysteine residue of Gi and Go proteins (3). ADP-ribosylation by cholera toxin promotes the dissociation of the Gs$\alpha\beta\gamma$ complex into Gsα and $\beta\gamma$ subunits and inhibits Gsα GTPase activity, causing persistent activation of adenylyl cyclase (4). ADP-ribosylation by pertussis toxin results in uncoupling of Gi proteins from receptors (5). In various cell types, many target proteins of endogenous ADP-ribosyltransferases have been found (2). Of particular interest, was the finding that the endogenous ADP-ribosylation of Gs proteins was correlated with increased adenylyl cyclase activity (6–11). We have previously proposed that the stimulation of Gi could enhance an endogenous ADP-ribosyltransferase acting on Gs in adipocytes (12); in the present study, we re-examined this possibility in permeabilized adipocytes. We report evidence for endogenous ADP-ribosylation of a 44 kDa protein which did not correspond to Gsα protein, but was nevertheless concomitant with an increase in adenylyl cyclase activity.

MATERIALS AND METHODS

Materials. [^{32}P]ATP, [^{32}P]NAD, [^{125}I]protein A, and anti-Gsα antiserum (RM1) were purchased from DuPont–New England Nuclear. Cholera toxin was from Sigma. Collagenase (CLS) was obtained from Worthington Biochemicals. 4,3-(Butoxy)-4-methoxybenzyl imidalidinone (RO 7-2956) was a gift from Hoffman-La Roche. Male Wistar rats (180 g) were from Depré (Saint Doulchard, France).

Cells permeabilization. White fat cells were isolated by collagenase digestion (13). Adipocytes were incubated with saponin (8 μg/ml) for 10 min in Krebs-Ringer bicarbonate buffer 0.1 M, pH 7.4, containing Ca^{2+} (1.3 mM) and 4% (w/v) fatty acid-free albumin (buffer A), washed and resuspended in the same buffer.

Determination of physiological parameters in fat cells. For lipolysis analysis, adipocytes (10^6 × cells/ml) were incubated for 30 min in the presence 10 μM isoproterenol. Free fatty acids (FFA) were extracted and determined as described (14). Glycerol was assayed as described (15). Triacylglycerols (TAG) were extracted as described (16). Viability of saponin-permeabilized cells, as determined by glu-

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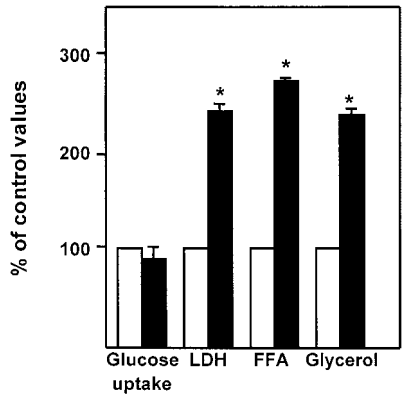

FIG. 1. Comparison of physiological parameters in control and saponin-permeabilized adipocytes. Adipocytes were permeabilized for 30 min with 8 μg/ml saponin (filled bars) or without (empty bars). The glucose uptake, the lactate dehydrogenase activity in extracellular medium (LDH) and lipolysis tested by measuring the release of free fatty acid (FFA) or glycerol were determined as described under Materials and Methods. The data are expressed as the percent of control values and are the mean ± SE of quadriplate determinations. Statistical significance versus control: * $P < 0.01$.

cose uptake, was assessed by using a peridochrom glucose assay kit (Boehringer Mannheim). Extracellular lactate dehydrogenase activity was measured as described (17). cAMP accumulation was measured by the radioimmunological method (18). Briefly, cells were incubated for 6 min in Krebs-Ringer bicarbonate supplemented with 1 mM RO 7-2956 (a cAMP-phosphodiesterase inhibitor) and 2 μg/ml adenosine deaminase, with or without agonists. FFA, glycerol and cAMP levels were expressed in function of TAG content of the cells.

[^{32}P]ADP-ribosylation of membrane proteins. For endogenous labelling (*in vivo* ADP-ribosylation), the cells were incubated with [^{32}P]NAD (1 μM) (10^7 cpm/ml) for 60 min at 37°C in 2 ml buffer A. At the end of the incubation period the washed cells were disrupted in 40 mM Tris-HCl, pH 7.4 containing 250 mM sucrose, 0.1 mM dithiothreitol and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin). The homogenate was centrifuged for 30 min at 30000*g*. The resulting pellet was resuspended in the homogenization buffer at a protein concentration of 1–2 mg/ml. For cholera toxin-catalysed ADP-ribosylation (*in vitro* ADP-ribosylation), aliquots of membrane fractions (200 μg protein) were incubated with 3 μM [^{32}P]NAD in the presence of activated cholera toxin (6 μg) as described (12). The labelled membranes were washed, solubilized, and analyzed by 10% SDS-polyacrylamide gel electrophoresis (10% SDS-PAGE).

Two-dimensional gel electrophoresis. Isoelectric focusing followed by SDS-PAGE (second dimension) was performed according to O'Farrel (19). Samples containing 200 μg of labelled proteins were applied to isoelectric focusing gels (first dimension) containing Ampholines, pH 3–10.

Western blot analysis of Gsα protein. Aliquots of membrane proteins (100 μg) were resolved on a 10% SDS-PAGE and transferred to nitrocellulose filters. The filters were then incubated overnight with RM/1 antiserum at a 1:500 dilution, and the immune complexes was revealed by incubation with ^{125}I-Protein A (2×10^5 cpm/ml) as previously described (20).

Adenylyl cyclase assays. Incubations were performed in the presence of various agents for 10 min at 32°C in 100 μl 40 mM Tris-HCl buffer (pH 7.4) containing 10–20 μg of membrane protein with 0.2 mM [^{32}P]ATP (10^5 cpm/assay), 2 mM $MgCl_2$ and an ATP-regenerating system as previously described (20). The [^{32}P]cAMP produced was quantified as described (21).

Statistical analysis. Statistical differences were calculated using the Student *t* test. A $P < 0.05$ was considered significant.

RESULTS

Permeabilization of rat adipocytes with saponin affected neither cell viability nor the adenylyl cyclase system. To allow NAD to penetrate into the cell, adipocytes were permeabilized with saponin. The permeabilization procedure was validated by measuring both lactate dehydrogenase (LDH) activity and the release of free fatty acids (FFA) and glycerol in the extracellular medium. LDH activity was significantly increased in permeabilized adipocytes compared to non-permeabilized fat cells. The amounts of FFA and glycerol were also higher in the incubation medium of permeabilized adipocytes. The cell viability was not affected by the treatment with saponin since glucose uptake was similar in permeabilized versus control cells (Fig. 1). Importantly, in Table 1, it is shown that basal and cAMP accumulation stimulated by either isoproterenol (a non-selective adrenergic receptor agonist), forskolin (a drug which stimulates directly the adenylyl cyclase) or cholera toxin (which activates adenylyl cyclase via activation of Gs proteins) was not affected by the treatment with saponin, indicating that the integrity of the adenylyl cyclase system was maintained in permeabilized cells.

Effect of NAD on cAMP accumulation and adenylyl cyclase activity in permeabilized adipocytes. Permeabilized adipocytes were incubated with NAD for 60 min, then crude membrane fractions were prepared and adenylyl cyclase activities were measured in the presence of various agonists: GTP, GTPγS, $[Al/F_4]^-$, isoproterenol, and forskolin. We found that NAD potentiated adenylyl cyclase activity stimulated by all agonists used (Table 2). Similarly, treatment of adipocytes with NAD (0.01–1 mM) increased in a dose-dependent manner cAMP accumulation stimulated by isoproterenol. At the highest concentration of NAD

TABLE 1

Cyclic AMP Accumulation in Control and Permeabilized Adipocytes

	cAMP (pmol/min/mmol TAG)	
Agonist	Control cells	Permeabilized cells
None	0.70 ± 0.04	0.67 ± 0.06
Forskolin (10 μM)	586.0 ± 47.0	482.7 ± 14.0
Isoproterenol (1 μM)	62.6 ± 6.0	51.5 ± 7.0
Cholera toxin (5 μg/ml)	2.26 ± 0.06	1.83 ± 0.04

Note. Control or saponin-permeabilized adipocytes were incubated for 6 min in the presence of forskolin, isoproterenol, or 2 h in the presence of cholera toxin. cAMP formation was determined as described under Materials and Methods. Each value represents the mean ± SE of quadriplate determinations.

TABLE 2

Effect of a Treatment of Permeabilized Adipocytes with NAD on Adenylyl Cyclase Activity

Agonist	Adenylyl cyclase activity (pmol/min/mg protein)	
	Control	NAD
GTP (10 μM)	10.2	11.4 (110)*
GTγS (100 μM)	71.6	112.2 (157)
$[Al/F_4]^-$ (NaF 10 mM + $AlCl_3$ 6 μM)	77.3	125.7 (163)
Isoproterenol (10 μM)	30.2	55.0 (182)
Forskolin (100 μM)	253.3	410.2 (162)

Note. Permeabilized adipocytes were incubated for 60 min in the presence or absence of NAD (1 μM). Then, adenylyl cyclase activities were measured on membrane fractions as described under Materials and Methods, in the presence of various agonists. Values are the mean of triplicate assays.

* Percent activity relative to that without NAD.

used (1 mM), a 1.4 fold increase in isoproterenol-stimulated cAMP accumulation was obtained when compared to cells not treated with NAD. L-Arginine, which was shown to be an ADP-ribosyl acceptor (1), suppressed the effect of NAD on cAMP accumulation (Fig. 2).

Endogenous ADP-ribosylated proteins in permeabilized adipocytes. To assess whether endogenous ADP-ribosylation of G proteins could account for NAD-induced potentiation of adenylyl cyclase activity, permeabilized adipocytes were incubated with [^{32}P]NAD (1 μM) for 60 min (*in vivo* ADP-ribosylation). Cells were then washed in buffer A and membrane fraction was prepared. In the same experiment, membranes proteins from permeabilized adipocytes non-incubated with NAD were subjected to ADP-ribosylation by cholera toxin in the presence of [^{32}P]NAD (*in vitro* ADP-ribosylation). Membrane proteins were resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane. After autoradiography of the membrane, we found that the major endogenous labelled protein had a Mw of 44 kDa on 10% SDS-PAGE (Fig. 3A, lane 1). It appeared clearly to migrate between both short (42 kDa) and long (46 kDa) isoforms of Gsα proteins ADP-ribosylated *in vitro* by cholera toxin (Fig. 3A, lane 2). Next, we checked whether the 44 kDa protein was recognized by the specific anti-Gsα antiserum RM/1. To this end, the samples were analysed by Western blot with the specific RM/1 antiserum. Both Gsα protein subunits of 42 and 48 kDa were recognized by the anti-Gsα antiserum (Fig. 3, lanes 3–4) and corresponded to Gsα proteins ADP-ribosylated *in vitro* by cholera toxin (lane 2). It was also clear that NAD treatment did not alter immunoreactivity of Gsα proteins (Fig. 3A, lane 3 versus lane 4). Importantly, the endogenous ADP-ribosylated protein was not recognized by the anti-Gsα antiserum (lane 3).

The labelling of the 44 kDa protein was reduced by increasing the concentrations of unlabelled NAD (0.5–100 μM) (Fig. 3B). Half maximum inhibition was obtained with 1 μM of NAD. Endogenous labelling of the 44 kDa protein was also inhibited in a dose-dependent manner by preincubation of cells with L-arginine methyl ester (LAME), an acceptor amino-acid for ADP-ribose moiety. Half-maximal inhibition was obtained with 2 mM LAME (Fig. 3C). The endogenous labelling of the 44 kDa protein was also blocked by meta-iodobenzylguanidine (MIBG) (10 μM), a high affinity substrate for mono-ADP-ribosyltransferase (22) (data not shown). When present in the incubation medium, GTPγS increased NAD-induced labelling of the 44 kDa protein. A maximal effect was observed with 1 μM GTPγS (Fig. 3D).

Analysis of the endogenous labelled 44 kDa protein on two dimensional gels. Isoelectric focusing and SDS-PAGE (2D gels) revealed that the endogenous labelled protein consisted of two polypeptides with a molecular mass of 44 kDa on 2D gels: one had an isoelectric point (pI) of approximately 7.0 and the other one of approximately 7.3 (Fig. 4). These characteristics were very different from those obtained for Gsα proteins ADP-ribosylated *in vitro* by cholera toxin. Indeed, on 2D gels, the substrates of cholera toxin consisted of two major spots which had a molecular mass of 42 kDa and 46 kDa respectively. Both 42 and 46 kDa bands yielded multiple spots in the isoelectric focusing dimension with pI ranging between 6.6 and 6.3. The

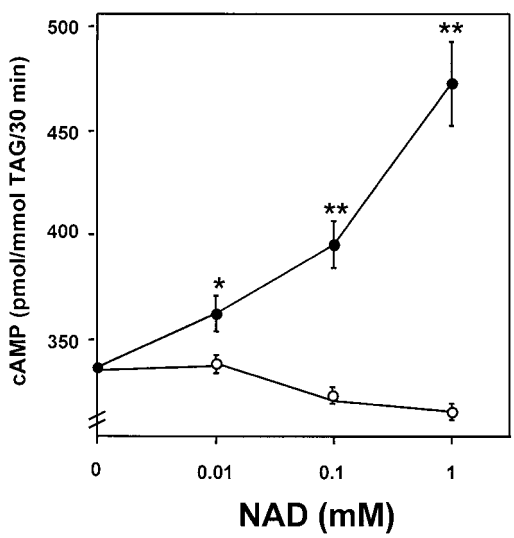

FIG. 2. Concentration-dependent effect of NAD on cAMP accumulation. Permeabilized adipocytes were incubated for 30 min in the presence of increasing concentrations of NAD without (●) or with L-arginine (75 mM) (○). Isoproterenol (1 μM) was added as an activator of adenylyl cyclase. cAMP formation was determined as described in Table 1. Values are the mean ± SE of quadriplate determinations. As compared to the respective control, * $0.02 < P < 0.05$, ** $P < 0.001$.

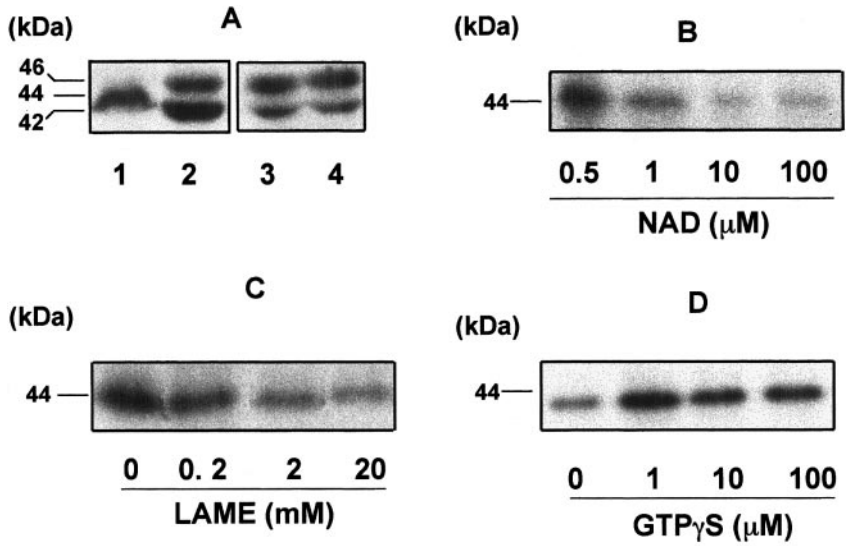

FIG. 3. Evidence for the presence of adipocyte membrane proteins ADP-ribosylated *in vivo*. (A) Adipocytes were permeabilized with saponin as in Fig. 1, then they were incubated for 60 min with [^{32}P]NAD (1 μM) for *in vivo* ADP-ribosylation. Membrane proteins were separated on 10% SDS/PAGE and transferred on nitrocellulose sheet and the labelled band was identified by autoradiography (lane 1). Membrane preparations of permeabilized cells were also ADP-ribosylated *in vitro* for 10 min in the presence of cholera toxin and [^{32}P]NAD and subjected to the same procedure (lane 2). Lanes 3 and 4 show the corresponding immunoreactivity of Gsα proteins in endogenously labelled membranes (lane 3) and in cholera toxin-labelled membranes (lane 4). (B) Autoradiograms of membrane proteins ADP-ribosylated *in vivo* in the presence of increasing concentration of NAD. (C) Autoradiograms of membrane proteins ADP-ribosylated *in vivo* in the presence of increasing concentration of L-arginine methyl ester (LAME). (D) Autoradiograms of membrane proteins ADP-ribosylated *in vivo* in the presence of increasing concentration of GTPγS. Each lane contained 100 μg of protein. These autoradiograms are representative of three separate experiments.

major ones having a pI of 6.4. Another smaller spot of pI = 6.3 was also detected between the two main labelled peptides. Different forms of Gsα proteins ADP-ribosylated by cholera toxin on 2D gels have also been described previously (23–25).

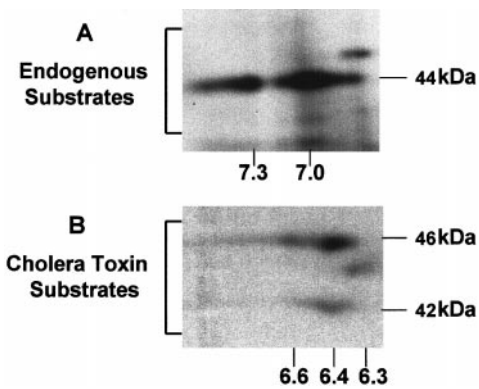

FIG. 4. Two-dimensional analysis of substrates for endogenous ADP-ribosylated proteins and for cholera toxin-mediated ADP-ribosylation of Gsα proteins. Membrane proteins were ADP-ribosylated endogenously (A) or by cholera toxin (B) as described in the legend to Fig. 3. Samples were focused (pH 3.5–10) and electrophoresed on 10% SDS-PAGE as described under Materials and Methods. Only a portion of autoradiograms is shown. The pH for the isoelectric focusing dimension decreases in a left-to-right manner. The isoelectric points of the predominant radiolabelled substrates are indicated.

Effect of NAD on the ADP-ribosylation of Gsα proteins by cholera toxin. Permeabilized adipocytes were incubated for various periods of time in the presence of unlabelled NAD, and crude membrane fractions were prepared. These membranes were then used in an ADP-ribosylation assay using [^{32}P]NAD and cholera toxin (*in vitro* assay). We hypothesized that if endogenous ADP-ribosylation of Gsα proteins occured during the preincubation with NAD, this should reduce the subsequent *in vitro* ADP-ribosylation of Gsα proteins by cholera toxin. This was not the case, as a two fold increase in cholera toxin-mediated ADP-ribosylation of Gsα proteins was observed in membrane fractions from adipocytes which were preincubated with NAD (Fig. 5). This result could not be related to an increased amount of Gsα proteins in membrane during the time course of NAD treatment since equal amounts of Gsα proteins were found over the time kinetic (Fig. 5).

DISCUSSION

We have presented evidence for the existence of an ADP-ribosyltransferase activity in rat adipocytes, thus confirming our initial findings (12). We demonstrated the endogenous ADP-ribosylation of one major protein in membrane fractions. This protein showed an apparent molecular mass of 44 kDa by SDS-PAGE, did not comigrate with the major cholera toxin-labeled pro-

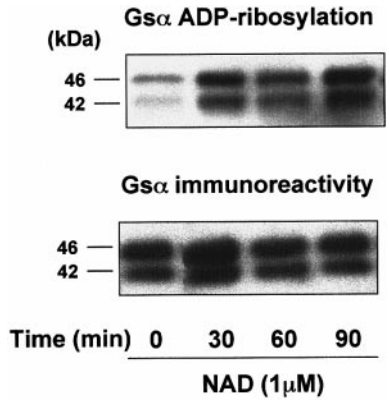

FIG. 5. Effect of NAD on cholera toxin-mediated ADP-ribosylation of Gsα protein. Permeabilized adipocytes were incubated for 10 to 90 min with (1 μM) NAD. Aliquots of membrane proteins (100 μg) were then ADP-ribosylated *in vitro* with cholera toxin and [^{32}P]NAD as described in the legend to Fig. 3. Autoradiogram of cholera toxin-mediated ADP-ribosylation of Gsα protein and Western blot analysis of Gsα protein levels in the same experiment are shown. This experiment is representative of three others.

teins in either 10% SDS-PAGE or two-dimensional electrophoresis and was not recognized by Gsα antibody. The fact that a treatment of permeabilized adipocytes with NAD did not reduce the subsequent *in vitro* ADP-ribosylation of Gsα protein subunits by cholera toxin further reinforced the view that the endogenous ADP-ribosylated 44 kDa protein was not related to Gsα proteins. Our results differ from the one described by Graves and McDonald where incubation of adipocyte plasma membrane with [^{32}P]NAD yielded ADP-ribosylation of proteins of 70, 65, 61 and 52 kDa (26). This difference might be due to the fact that we have used permeabilized adipocytes whereas the cited authors have used plasma membrane-enriched fractions. One might conclude that the integrity of the cell is required to achieve adequate endogenous ADP-ribosylation of the 44 kDa protein.

We have characterized some properties of the 44 kDa protein in adipocytes. Its labelling was increased in the presence of GTPγS suggesting that it was a GTP-binding protein. We have also excluded that the 44 kDa protein was a Gi protein because a pretreatment of adipocytes with pertussis toxin did not affect the subsequent endogenous labelling of the 44 kDa protein (data not shown) and because arginine was the residue of the 44 kDa protein which was covalently modified.

The interesting finding is that despite the fact that NAD did not induce endogenous ADP-ribosylation of Gsα in permeabilized adipocytes, NAD potentiated the adenylyl cyclase response to isoproterenol or to agonists activating the adenylyl cyclase cascade at a post receptor level. Thus, our results obtained with adipocytes were different from those presented by others using membrane preparations of rabbit luteal cells (8), human platelets (7, 9), chick spleen cells (10), and neuroblastoma glioma cells (11). These authors reported endogenous ADP-ribosylation of Gsα following incubation with NAD which correlated with NAD-induced potentiation of adenylyl cyclase activity.

In permeabilized adipocytes, we do not know yet how NAD treatment modified the adenylyl cyclase response but some evidences suggest that nonetheless it could be due to a change in the functional activity of Gs proteins. Indeed, NAD treatment increased the adenylyl cyclase response to agonist acting directly on Gs proteins (GTPγS and $[Al/F_4]^-$) without affecting the amount of Gs proteins. It might be that NAD induced change in the dissociation of Gs protein into α and βγ subunits. However, since free α subunit of Gs and the αβγ trimer can be equally good substrates for cholera toxin (4), our data showing that a treatment of permeabilized adipocytes with NAD increased the subsequent *in vitro* ADP-ribosylation of Gsα subunits by cholera toxin do not allow to conclude on this hypothesis. But this unexpected finding might further support the view that NAD treatment could have modified the function of Gs protein. Indeed, cholera toxin-induced ADP-ribosylation of Gsα subunits has been used to evaluate the function of Gs proteins. For instance, we have shown that a decreased ability of cholera toxin to ADP-ribosylate Gs protein in thyroid cells correlated with an impairment of the functional activity of Gs proteins (27).

In summary, we do not know the identity of this 44 kDa endogenous ADP-ribosylated protein and also whether there is a link between its endogenous labelling and the increased adenylyl cyclase activity. The possibility remains that the potentiation effect of NAD on the adenylyl cyclase system was due to an effect on Gs protein which was not related to endogenous ADP-ribosylation of Gs proteins.

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