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Enhancement by GOSPEL protein of GAPDH aggregation induced by nitric oxide donor and its inhibition by NAD$^+$

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Glyceraldehyde-3-phosphate dehydrogenase's (GAPDH's) competitor of Siah Protein Enhances Life (GOSPEL) is the protein that competes with Siah1 for binding to GAPDH under NO-induced stress conditions preventing Siah1-bound GAPDH nuclear translocation and subsequent apoptosis. Under these conditions, GAPDH may also form amyloid-like aggregates proposed to be involved in cell death. Here, we report the *in vitro* enhancement by GOSPEL of NO-induced GAPDH aggregation resulting in the formation GOSPEL-GAPDH co-aggregates with some amyloid-like properties. Our findings suggest a new function for GOSPEL, contrasting with its helpful role against the apoptotic nuclear translocation of GAPDH. NAD$^+$ inhibited both GAPDH aggregation and co-aggregation with GOSPEL, a hitherto undescribed effect of the coenzyme against the consequences of oxidative stress.

Keywords: nicotinamide adenine dinucleotide; nitric oxide; nitrosative stress; oxidative stress; protein aggregation

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the reversible formation of 1,3-bisphosphoglycerate from glyceraldehyde-3-phosphate and inorganic phosphate with a concomitant reduction of NAD$^+$ to NADH. A large body of evidence indicates that in addition to being involved in glycolysis, GAPDH has multiple functions in a variety of biochemical processes [1], including a proapoptotic role under oxidative stress conditions. Apoptotic stimuli lead to the generation of nitric oxide (NO) and S-nitrosylation of GAPDH. The S-nitrosylation of GAPDH at the active site cysteine augments its binding to Siah1, an E3-ubiquitin-ligase whose nuclear localization signal mediates the nuclear translocation of the GAPDH-Siah1 complex. Inside the nucleus, GAPDH stabilizes Siah1 promoting the degradation of selected nuclear targets of Siah1 and finally apoptosis [2]. A protective effect against apoptosis caused by the GAPDH-Siah1 complex nuclear translocation was described in rat for a protein named GOSPEL (GAPDH's competitor of Siah Protein Enhances Life) [3]. The S-nitrosylation of GOSPEL promotes its binding to S-nitrosylated GAPDH in competition with Siah1, retaining GOSPEL-bound GAPDH in the cytosol and preventing the cytotoxic effect [3].

In vitro S-nitrosylation of GAPDH also results in the formation of insoluble aggregates with amyloid fibril characteristics due to intermolecular disulfide bonding [4,5]. Amyloid-like aggregation of S-nitrosylated GAPDH was also produced under oxidative stress

Abbreviations
BSA, bovine serum albumin; G3P, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GOSPEL, GAPDH's competitor of Siah Protein Enhances Life; GSNO, S-nitrosoglutathione; NOR3, (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide; RLP1, RILP-like protein 1; TEM, transmission electron microscopy; ThT, Thioflavin-T; TPI, triose phosphate isomerase.

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conditions promoting cell death [4]. A high correlation was observed between the toxic role of GAPDH under these conditions and the abnormal enzyme aggregation [5].

The catalytic site cysteines of the native GADPH tetramer are not able to form disulfide bonds owing to distance constraints. The proposed model for GADPH aggregation elicited by oxidative stress involves a conformational change that exposes other cysteines for the formation of disulfide-bonded oligomers and insoluble aggregates [4]. The proposal also considers the possible involvement of an aggregation mechanism other than disulfide bonding, due to the appearance of SDS-stable GAPDH oligomers resistant to DTT, both *in vitro* and in cells. Recently reported evidence in support of such a mechanism is based on the observation that the NO-donor NOR3 produced the oxidation of specific GAPDH methionine, tyrosine and tryptophan residues. In particular, the conversion of Met46 into Met46-sulfoxide was considered the primary event in the free-radical induced oxidation [6].

Both nuclear translocation of Siah1-bound GAPDH and GAPDH self-aggregation are involved in cytotoxic stress, the translocation produced as an initial event triggered by low stress-induced oxidation and the formation of aggregates in the cytoplasm promoted by massive levels of oxidative stress [5]. No studies have been reported on the effect of GOSPEL in the latter event.

In the present work, we describe for the first time the *in vitro* enhancement by GOSPEL of the GAPDH aggregation induced by the NO-donor NOR3, suggesting a possible new function for this protein. Inhibition of the aggregation produced by NAD^+ is also reported.

Materials and methods

Materials

Escherichia coli strain Rosetta(DE3) and vector pET15b were from Novagen. Human GAPDH (GenBank number BC029618), GOSPEL (GenBank number BC089444), and Triosephosphate isomerase (TPI, GenBank number BC015100) cDNA clones were from Open Biosystems. HisTrap FF columns were from GE Healthcare. Primary antibodies and horseradish peroxidase-conjugated secondary antibody were from Abcam and dye labeled secondary antibodies were from LI-COR. Except where indicated, all other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Mutagenesis, expression, and purification

cDNA of human GAPDH, GOSPEL, and TPI were inserted into the expression vector pET15b, downstream of the His_6-tag encoding sequence of the plasmid. The resulting constructs were used as templates to generate the mutants GAPDH M46L, GAPDH C152S, and GOSPEL C47S by site-directed mutagenesis utilizing the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

His_6-wild-type and -mutant proteins were expressed in *Escherichia coli* strain Rosetta(DE3) and purified by affinity chromatography on HisTrap FF columns (1 mL). Fractions containing the protein of interest were pooled, incubated with 1 mM DTT at 4 °C overnight, desalted with 20 mM Tris-HCl buffer pH 7.5 containing 1 mM EDTA and concentrated. Protein concentration was determined based on calculated extinction coefficients at 280 nm of 30 000 $M^{-1}·cm^{-1}$, 16 960 $M^{-1}·cm^{-1}$ and 33 460 $M^{-1}·cm^{-1}$ for GAPDH, GOSPEL, and TPI, respectively.

Protein solution turbidity

Protein aggregation was induced as described by Nakajima *et al.* [4]. Briefly, GAPDH (16.5 μM i.e. 0.6 $mg·mL^{-1}$) was incubated with 100 μM NOR3 in 50 mM Tris-HCl buffer pH 8.0, 150 mM NaCl, 1 mM EDTA, and 5% glycerol (incubation buffer) at 37 °C for the times indicated in the figure legends. NOR3 used in all the experiments was freshly prepared in DMSO. Aggregation experiments in the presence of GOSPEL, GOSPEL C47S, TPI, or bovine serum albumin (BSA), were carried out using equimolar amounts of GAPDH and the corresponding protein, unless otherwise indicated. The effect of NAD^+, glyceraldehyde 3-phosphate, inorganic phosphate and deprenyl in protein aggregation was analyzed including each compound in the incubation mixture 30 min before the addition of NOR3. Aggregation was measured by turbidity at 405 nm using an Epoch microplate reader (Biotek, Winooski, VT, USA).

Circular dichroism analysis of GOSPEL

Glyceraldehyde-3-phosphate dehydrogenase's competitor of Siah Protein Enhances Life (5 $mg·mL^{-1}$) was incubated at 37 °C with or without 1 mM NOR3 for 6 h in incubation buffer. After buffer exchange with 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA, far-UV circular dichroism (CD) spectra of the proteins were recorded between 190 nm and 250 nm using a JASCO-810 spectropolarimeter (JASCO). Secondary structure analysis was performed with the CDSSTR algorithm [7] from DICHROWEB [8–10].

Thioflavin-T fluorescence assay

Fifty microliter aliquots from each sample were mixed with 450 μL of 200 μM Thioflavin-T (ThT). Fluorescence was measured using a FluoroMax-P fluorimeter (Horiba Jobin Yvon, Edison, NJ, USA) with excitation and emission wavelengths of 450 nm and 482 nm, respectively.

Gel electrophoresis and two-color fluorescent western blot

Aggregate samples pelleted by centrifugation were washed twice with incubation buffer and resuspended in sample buffer containing 1% SDS with or without 300 mM DTT. Aliquots of the solubilized precipitates were subjected to SDS/PAGE. Western blot analysis was performed with the primary antibodies anti-GAPDH rabbit polyclonal and anti-GOSPEL mouse polyclonal that were detected simultaneously by the secondary antibodies IRDye® 680 goat anti-mouse IgG and IRDye® 800 goat anti-rabbit IgG using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). The fluorescent signals were quantified by densitometric analysis using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay

The effect of NAD^+ in GOSPEL-GAPDH interaction was analyzed by Enzyme-linked immunosorbent assay (ELISA). Briefly, wells of a polystyrene ELISA plate were coated overnight at 4 °C with GOSPEL (35 $\mu g \cdot mL^{-1}$) and blocked with blocking buffer (20 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20, and 1.5% non-fat milk). GAPDH (35 $\mu g \cdot mL^{-1}$) alone or with NAD^+ was then added to the wells and incubated for 1 h at 37 °C after the addition of S-nitrosoglutathione (GSNO). The wells were washed with washing buffer (20 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.05% Tween 20), incubated with anti-GAPDH rabbit polyclonal antibody for 1 h and washed again. After incubation with horseradish peroxidase (HRP)-conjugated antibody the bound HRP was monitored by color development of *o*-phenylenediamine/H_2O_2 with absorbance at 490 nm. The values were corrected by subtracting the absorbance of nonspecifically bound GAPDH.

Congo red birefringence and electron microscopy

Congo red staining was carried out according to Nakajima *et al.* [4]. Briefly, 100 µL aliquots of 24-h aggregate suspensions prepared as described above were mixed with 900 µL of Congo red solution (25 $\mu g \cdot mL^{-1}$). After 30 min, the mixtures were centrifuged and the pellets obtained were resuspended in 50 µL water. Samples (10 µL) were air dried on glass slides and finally analyzed in bright and polarized light using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany). For transmission electron microscopy (TEM) analysis, 5 µL aliquots of the protein aggregated suspensions were adsorbed onto 200-mesh formvar/carbon-coated nickel grids for 10 min. The grids were then washed with water and negatively stained with 2% uranyl acetate. TEM images were collected using a JEM-1220 electron microscope (JEOL, Tokyo, Japan).

Statistical analysis

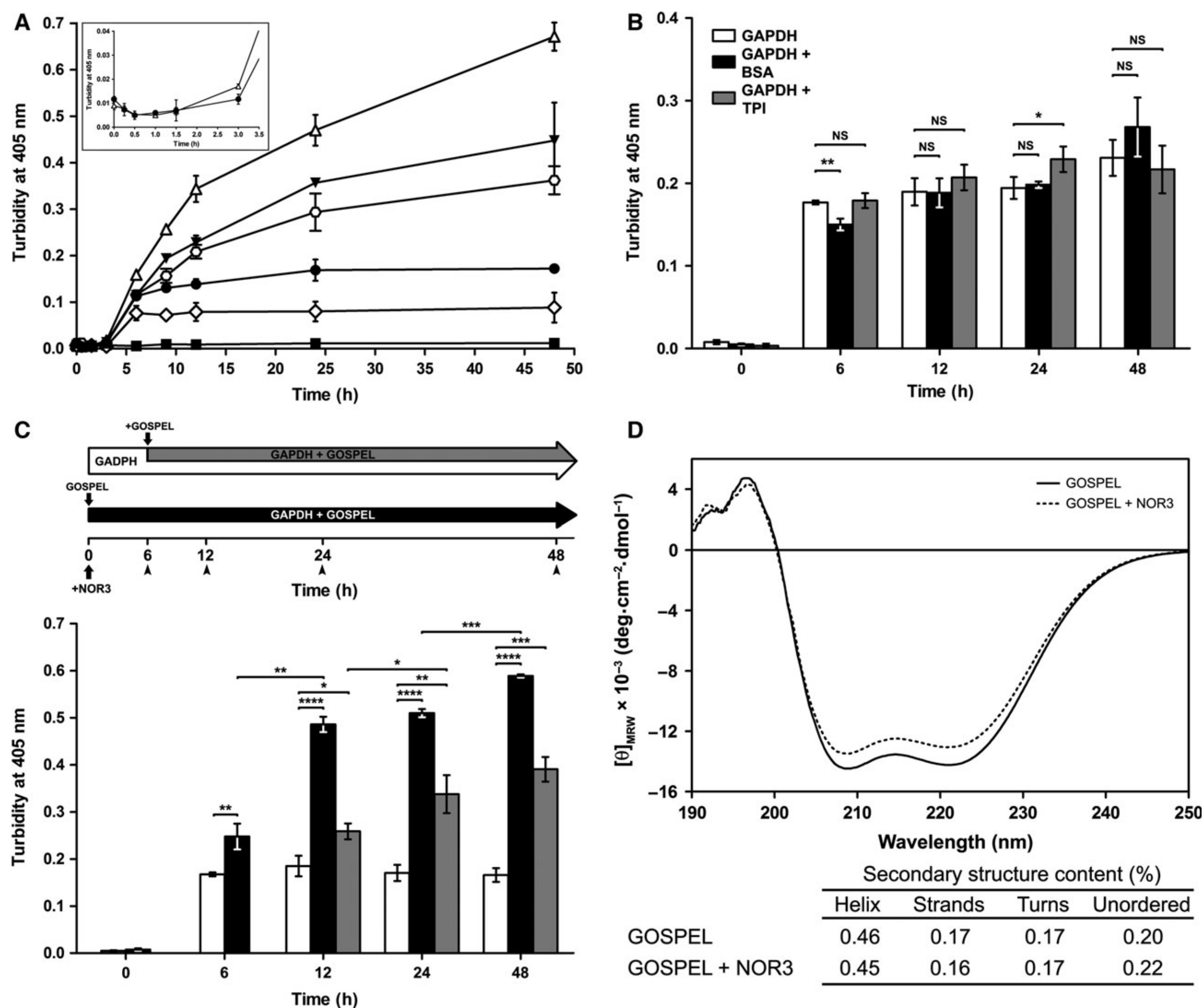
Data are presented as mean ± SD. Statistical significance was determined using one-way ANOVA with Bonferroni post hoc test. A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

GOSPEL enhances oxidation-induced GAPDH aggregation

Rat GOSPEL was identified as a protein interactor of GAPDH in a yeast two-hybrid analysis [3]. This study described an enhancement of GOSPEL binding to GAPDH under conditions that led to S-nitrosylation of both proteins. In a database search for rat GOSPEL homologs in humans, we identified a protein named RLP1 (RILP-like protein 1), which is 93% identical to GOSPEL. We determined that like rat GOSPEL, human RLP1 has the capacity to be S-nitrosylated and to bind to S-nitrosylated GAPDH [11], for which reason we refer to it as human GOSPEL.

It has been described that incubation of rat GAPDH with the NO-donor NOR3 leads to the formation of disulfide-bonded oligomers and a time-dependent increase in the solution turbidity, due to the aggregation of insoluble high molecular weight GADPH oligomers [4]. Using purified recombinant human proteins, we focused our studies on the effect of GOSPEL in the NOR3-induced GADPH aggregation. The presence of GOSPEL in the incubation mixture enhanced both the rate and extent of turbidity (Fig. 1A). However, the lag time that preceded the GAPDH turbidity increase was not modified by GOSPEL (Fig. 1A, inset), consistent with aggregation occurring after the proposed GAPDH misfolding triggered by oxidation of Met46 [6]. Evidence in support of Met46 being involved in NO-induced GAPDH oxidation was obtained when M46L mutant replaced wild-type GAPDH, resulting in a reduction in the rate and extent of the turbidity increase, both in the absence and presence of GOSPEL (Fig. 1A). GOSPEL did not induce the aggregation of C152S, the GAPDH mutant lacking the active site cysteine, which is unable to aggregate by incubation with NOR3 [4] (results not shown). No turbidity of the GOSPEL solution was observed when incubated with NOR3 in the absence of GAPDH (Fig. 1A) or in the presence of preformed GAPDH aggregates previously washed with incubation buffer (result not shown). The effect of GOSPEL was specific since no effect was observed when it was replaced by albumin, which can also be S-nitrosylated



	Secondary structure content (%)			
	Helix	Strands	Turns	Unordered
GOSPEL	0.46	0.17	0.17	0.20
GOSPEL + NOR3	0.45	0.16	0.17	0.22

Fig. 1. Effect of GOSPEL on *in vitro* NO-induced GAPDH aggregation. (A) Time course of NOR3-induced GAPDH solution turbidity increase in the presence of GOSPEL. GAPDH (●), GAPDH M46L (◊) GOSPEL (■), and mixtures of GAPDH with equimolar amounts of GOSPEL (△), GOSPEL C47S (▼), and of GAPDH M46L with GOSPEL (○) were incubated with 100 μM NOR3 as described in Materials and methods. At the indicated times, the turbidity of each incubation mixture was measured at 405 nm. The inset is an expanded image of the first 3 h of incubation showing the lag phase. (B) GAPDH (white bars) and mixtures of GAPDH with equimolar amounts of BSA (black bars) and TPI (gray bars) were incubated and analyzed as detailed in (A). (C) GADPH was incubated with NOR3 in the presence and absence of GOSPEL as in (A). The incubation mixture lacking GOSPEL was separated into halves at 6-h incubation, GOSPEL was added to one half and incubation continued as indicated in the top of the figure. Turbidity was measured at 0, 6, 12, 24, and 48 h. (D) Far-UV CD spectra of wild-type GOSPEL before (solid line) and after NOR3 treatment for 6 h (dotted line). The secondary structure content expressed as a percentage is summarized in the table below. The values plotted in (A)-(C) are the means ± SD of triplicate determinations. $*P < 0.05$, $**P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. *NS*, not significant.

[12,13], or by triosephosphate isomerase (TPI), another glycolytic enzyme which was found S-nitrosylated in Alzheimer's disease brain specimens [14] and identified as a GAPDH interactor [15] (Fig. 1B). Replacement of wild-type GOSPEL by the mutant in which its unique cysteine (Cys47) has been substituted by serine, also resulted in turbidity increase, although at a lower rate and extent (Fig. 1A). The addition of GOSPEL to the GAPDH aggregate suspension at the time the turbidity level reached a plateau produced a further time-dependent turbidity rise, although at a lower rate and extent than the addition of GOSPEL at the beginning of the assay (Fig. 1C). This indicates that in the absence of GOSPEL, some of the NO-oxidized GAPDH remains in a soluble form and is able to be recruited by the added GOSPEL into insoluble aggregates. GOSPEL-

promoted aggregation might thus commence with the binding of GOSPEL to disulfide-bound GADPH dimer and other lower oligomeric forms. Thereafter, the GOSPEL-GAPDH dimer/oligomer complex could act as a seed for further GOSPEL and GAPDH aggregation by disulfide-crosslinking involving any of the other two GAPDH cysteines (Cys156 and Cys247). The far-UV CD spectrum of NOR3-treated GOSPEL exhibited only a minor decrease in intensity between 205 and 230 nm relative to the native protein, probably caused by a slight increase in the unordered structure content (Fig. 1D). This result indicates that any oxidative modification undergone by GOSPEL during NOR3 incubation does not produce a significant change in its conformation. Although CD studies showed that NOR3 induces some conformational changes in GAPDH [4,5], gel filtration analysis of the remaining soluble protein in the NOR3 3-h incubated mixture showed that GAPDH was mostly present in a tetrameric form (Fig. S1).

Glyceraldehyde-3-phosphate dehydrogenase activity was not significantly modified by the presence of GOSPEL (Fig. S2A) as previously reported [3]. Instead, NOR3 treatment produced its complete inactivation after 15 min of incubation (Fig. S2B), at which time the protein aggregates are still not detectable by turbidity measurements (Fig. 1A, inset). This effect was also observed in the presence of GOSPEL (Fig. S2B).

Characterization of the GAPDH aggregates formed in the presence of GOSPEL

The increment produced by GOSPEL in NOR3-induced turbidity was dependent on the molar proportion of the proteins (Fig. 2A). Reducing SDS/PAGE analysis of the insoluble aggregates isolated by centrifugation revealed that GOSPEL co-aggregated with GAPDH (Fig. 2B). This analysis also showed that the turbidity increment observed with GOSPEL was due not only to its co-aggregation with GAPDH but also to the augmented aggregation of GAPDH (Fig. 2B). In fact, GOSPEL and GAPDH content in the insoluble aggregates increased as a function of the molar proportion between both proteins in the incubated solution (Fig. 2C).

NOR3-induced turbidity of the GAPDH solution, in the absence and presence of GOSPEL, was not affected by 1% Triton X-100 or 300 mM DTT but was clarified by the addition of 1% SDS and SDS-containing DTT (Fig. 3A). The resistance of the aggregates to DTT in the absence of SDS could be attributed to non-covalent interactions. Although SDS was able to clarify GAPDH and GAPDH-GOSPEL suspensions, the complete dissociation of the high molecular weight oligomeric species forming the aggregates required the presence of DTT, as observed by SDS/PAGE (Fig. 3B). As previously described [4], GAPDH insoluble aggregates formed in the absence of GOSPEL were resolved into GAPDH monomer and high molecular weight species retained at the top of the stacking gel and others that hardly entered the upper zone of the resolving gel (Fig. 3B, −DTT, lane 1), when analyzed by SDS/PAGE under nonreducing conditions. Upon reducing SDS/PAGE, most of these high molecular weight species disappeared with a concomitant increase in monomeric GAPDH (Fig. 3B, +DTT, lane 1). In the presence of GOSPEL, the nonreducing SDS/PAGE analysis showed a pattern similar to that of GAPDH without GOSPEL, except for a low proportion of monomeric GOSPEL (Fig. 3B, −DTT, lane 2). Reducing SDS/PAGE analysis of GAPDH-GOSPEL co-aggregates showed that the species not entering the stacking and resolving gel and a major part of those running in the upper zone of the resolving gel disappeared, with a simultaneous augmentation of GAPDH and GOSPEL (Fig. 3B, +DTT, lane 2). When the cysteine-free GOSPEL C47S mutant replaced the wild-type protein, nonreducing and reducing SDS/PAGE analysis of the co-aggregates showed a similar pattern, except for the lack of augmentation of the GOSPEL band (Fig. 3B, −/+DTT, lane 3). This result is consistent with co-aggregation of GOSPEL with GAPDH by both DTT-labile disulfide-linkage and SDS-labile noncovalent association.

Glyceraldehyde-3-phosphate dehydrogenase aggregates formed in the absence and presence of GOSPEL or GOSPEL C47S were further analyzed using Thioflavin-T (ThT) binding, Congo red birefringence and transmission electron microscopy (TEM). As shown in Fig. 4, there was an increase in the ThT fluorescence intensity in the aggregates formed after the incubation with NOR3, indicating that they could have some amyloid-like structures. As expected, GOSPEL and GOSPEL C47S, which remained soluble after the incubation with NOR3, showed no ThT fluorescence increase. Analyzed by polarized light microscopy after Congo red staining, only part of the GAPDH and GAPDH-GOSPEL aggregates exhibited the yellow green birefringence that characterizes ordered amyloid-like aggregates (Fig. 5, upper panels). However, TEM analysis of the two samples revealed that neither of them showed the typical fibrillar structure of amyloid aggregates (Fig. 5, lower panels). An analogous observation of amyloid aggregates lacking fibrillar structure

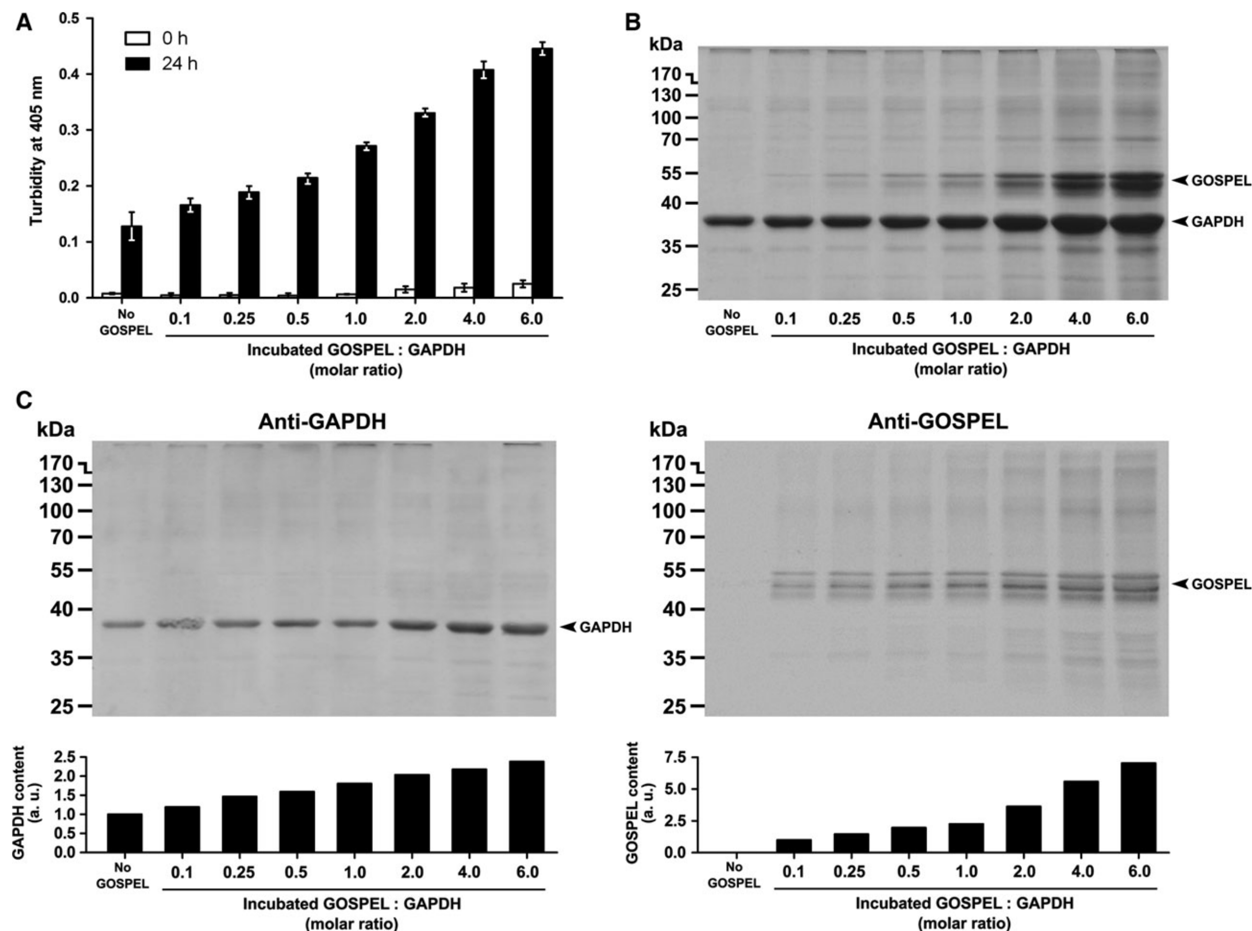


Fig. 2. GOSPEL:GADPH ratio dependence of the GAPDH solution turbidity enhancement and formation of insoluble GOSPEL-GAPDH co-aggregates. (A) GAPDH was incubated for 24 h with increasing amounts of GOSPEL in the presence of 100 μM NOR3. The turbidity of each mixture was determined at 405 nm, before (white bars) and after (black bars) incubation with the NO-donor. (B) The insoluble material present in the incubation mixtures was recovered by centrifugation according to the protocol detailed in Materials and methods and aliquots corresponding to 100 μL of the total incubated mixture were analyzed by reductive SDS/PAGE on gels stained with Coomassie blue. (C) The content of GAPDH and GOSPEL in the samples (30 μL of the total incubated mixture) was evaluated by western blot using specific antibodies. Relative levels of each protein were determined by densitometry.

was recently reported by Samson *et al.* [6], working with human wild-type and mutant GAPDH aggregates. The electron micrographs of GAPDH and GAPDH-GOSPEL aggregates showed that both are amorphous although they exhibited a clearly different morphology, the former being large and branched and the latter having a uniform granular appearance (Fig. 5, lower panels). The presence of mixtures of amorphous and fibrillar structures is not altogether uncommon and has also been described by others [16–20]. The results obtained with the GAPDH-GOSPEL C47S co-aggregates using both microscopy techniques are very similar to those observed for the wild-type GOSPEL-containing co-aggregates (results not shown).

Effect of NAD+, glyceraldehyde-3-phosphate, and inorganic phosphate on the oxidation-induced aggregation of GADPH and GAPDH-GOSPEL

The manner in which NAD^+, the GAPDH coenzyme, and glyceraldehyde-3-phosphate (G3P), and inorganic phosphate (Pi), its substrates, potentially affect NOR3-induced aggregation in the presence and absence of GOSPEL was also analyzed. While G3P and Pi had no significant effect on GAPDH aggregation with or without GOSPEL, pre-incubation of GAPDH and GAPDH-GOSPEL solutions with NAD^+ resulted in a coenzyme concentration-dependent inhibition of the turbidity (Fig. 6A). At a concentration of 500 μM, which is near the physiological concentration found in

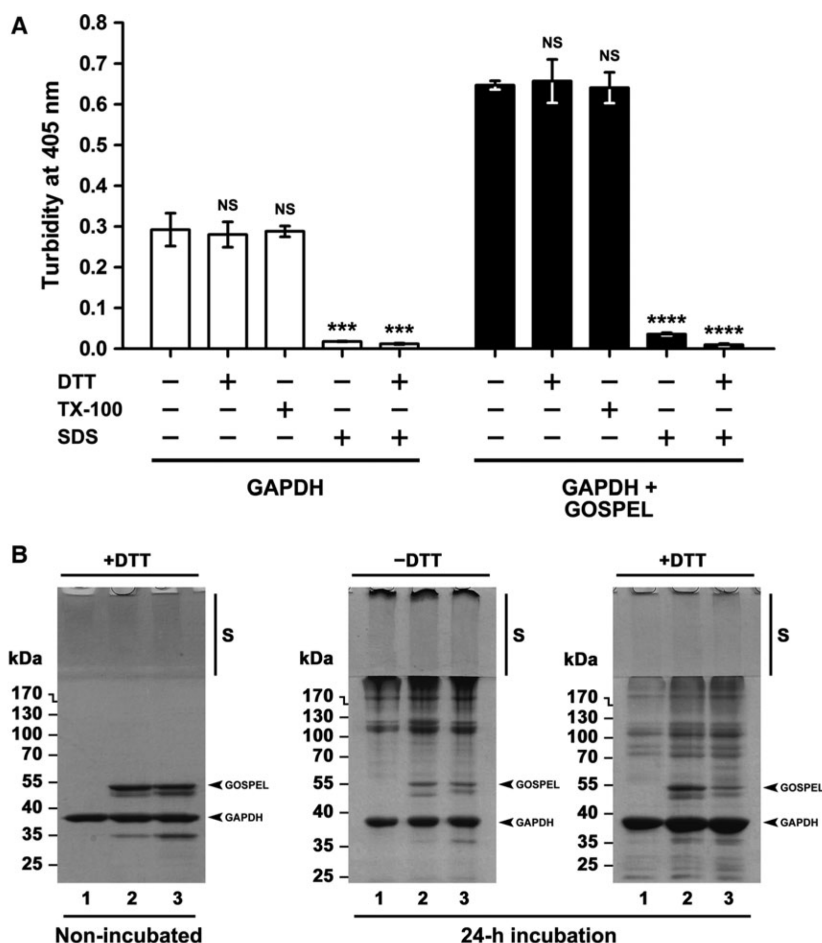


Fig. 3. Response of GAPDH and GOSPEL-GAPDH protein aggregates to detergent and reducing treatment. (A) GAPDH and an equimolar mixture of GAPDH and GOSPEL were incubated with 100 μM NOR3 for 24 h as described in Materials and methods. Aliquots of the incubates were treated with 300 mM DTT, 1% Triton X-100 (TX-100), 1% SDS, or a combination of 300 mM DTT and 1% SDS for 1 h and the turbidity of the incubated mixtures was monitored at 405 nm. Results are expressed as means ± SD of triplicate determinations. ***$P < 0.001$ and ****$P < 0.0001$ vs control without additives. *NS*, not significant. (B) SDS/PAGE under nonreduced (−) and reduced (+) conditions of the insoluble aggregates obtained after incubation of GAPDH (1) and equimolar quantities of GAPDH and GOSPEL (2) or GAPDH and GOSPEL C47S (3) with 100 μM NOR3 for 24 h. Aggregates were precipitated and washed by centrifugation as detailed in Materials and methods. Aliquots of the resuspended precipitate equivalent to 60 μL of the total incubated mixture were analyzed by SDS/PAGE in the absence (−) or presence (+) of 300 mM DTT. As control, aliquots of the reaction mixtures before incubation were also subjected to reducing SDS/PAGE. The position of GAPDH and GOSPEL in the gel is indicated with arrows. S denotes the stacking gel.

human cells (~500–600 μM) [21], NAD^+ caused a reduction in turbidity with and without GOSPEL of 70% and 88%, respectively. A decrease in turbidity was also observed when GOSPEL was added to the GAPDH suspension at the turbidity plateau (as in Fig. 1C) in the presence of the coenzyme (Fig. 6B). As previously indicated, S-nitrosylation of GAPDH and GOSPEL enhances the interaction between them [3]. In order to determine the effect of NAD^+ on this interaction, we promoted the S-nitrosylation of both proteins with the NO-donor S-nitrosoglutathione (GSNO), which induces GAPDH aggregation at slower rate than NOR3 (results not shown), and analyzed their binding in the presence and absence of the coenzyme. As indicated by ELISA assay, NAD^+ inhibited GAPDH-GOSPEL interaction (Fig. 6C). No binding was detected in the absence of GSNO treatment (result not shown). Since S-nitrosylated GAPDH is irreversibly modified by the covalent attachment of NAD^+ to its active site cysteine, becoming permanently inactivated [22–25], the replacement of NO by NAD^+ could explain the lower binding to GOSPEL. The analysis of the GAPDH crystal structure [26] indicates that some residues of the region proposed to be essential for its interaction with GOSPEL (amino acids 80–120) [3] are part of the NAD^+ binding pocket

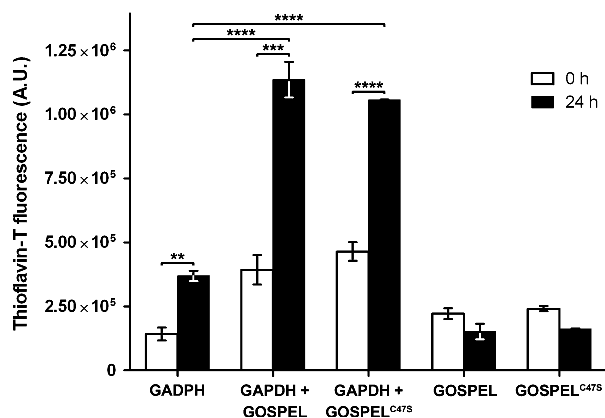


Fig. 4. Thioflavin-T binding analysis of GAPDH, GOSPEL-GAPDH and GOSPEL C47S-GAPDH aggregates. GAPDH and equimolar mixtures of GOSPEL-GAPDH and GOSPEL C47S-GAPDH were incubated for 24 h with 100 μM NOR3. Aliquots of each sample, taken before (white bars) and after (black bars) incubation with the NO-donor, were assessed for Thioflavin-T fluorescence as described in Materials and methods. Samples of GOSPEL and GOSPEL C47S were also assayed. Results are presented as the means ±SD of triplicate measurements. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

(Fig. S3), thus the presence of the coenzyme could modify the position of this domain interfering with the interaction. The latter result suggests that the inhibition by NAD^+ of the aggregation in the presence of GOSPEL observed in Fig. 6A,B, can be caused not only by the blockade of Cys152 but also by the interference of the interaction between the proteins. GOSPEL would only recruit the NAD-free fraction of GAPDH before it is modified by the coenzyme. Our results suggest that although NAD^+ causes loss of GAPDH activity under oxidative stress conditions, it might nevertheless protect the enzyme from aggregation, an event that could lead to more damaging consequences than the loss of activity since the high GAPDH content in cells would assure sufficient active enzyme to sustain its metabolic function.

Deprenyl (selegiline), a drug used in the treatment of Parkinson disease, binds to GAPDH, prevents its S-nitrosylation and blocks its nuclear translocation by inhibiting the binding to Siah1 [27]. As described for the aggregation of GAPDH both *in vitro* and in cells [5], the NOR3-induced aggregation of GAPDH in the presence of GOSPEL was not affected by this compound (results not shown).

Conclusions

The current results reveal a hitherto unreported property of GOSPEL, the ability to favor GAPDH aggregation by the formation of reduction-resistant GOSPEL-GAPDH co-aggregates under oxidative conditions. This would imply a potential new role for this protein in total contrast to the prevention of cell apoptosis elicited by nuclear translocation of Siah1-bound GAPDH. An interesting possibility to explore is whether the GOSPEL effect described here, in association or not with modified proteins other

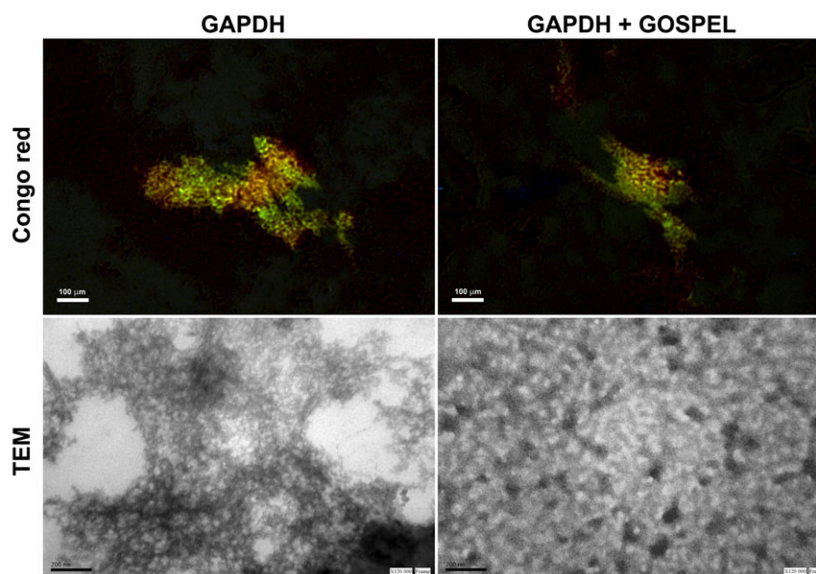


Fig. 5. Nonfibrillar amyloid-like structures of GAPDH and GOSPEL-GAPDH aggregates. Twenty-four-hour aggregates of GAPDH (left panels) and GOSPEL-GAPDH (right panels) obtained by incubation with 100 μM NOR3 were examined by microscopy under polarized light after Congo red staining (upper panels) and by transmission electron microscopy (lower panels), as described in Materials and methods.

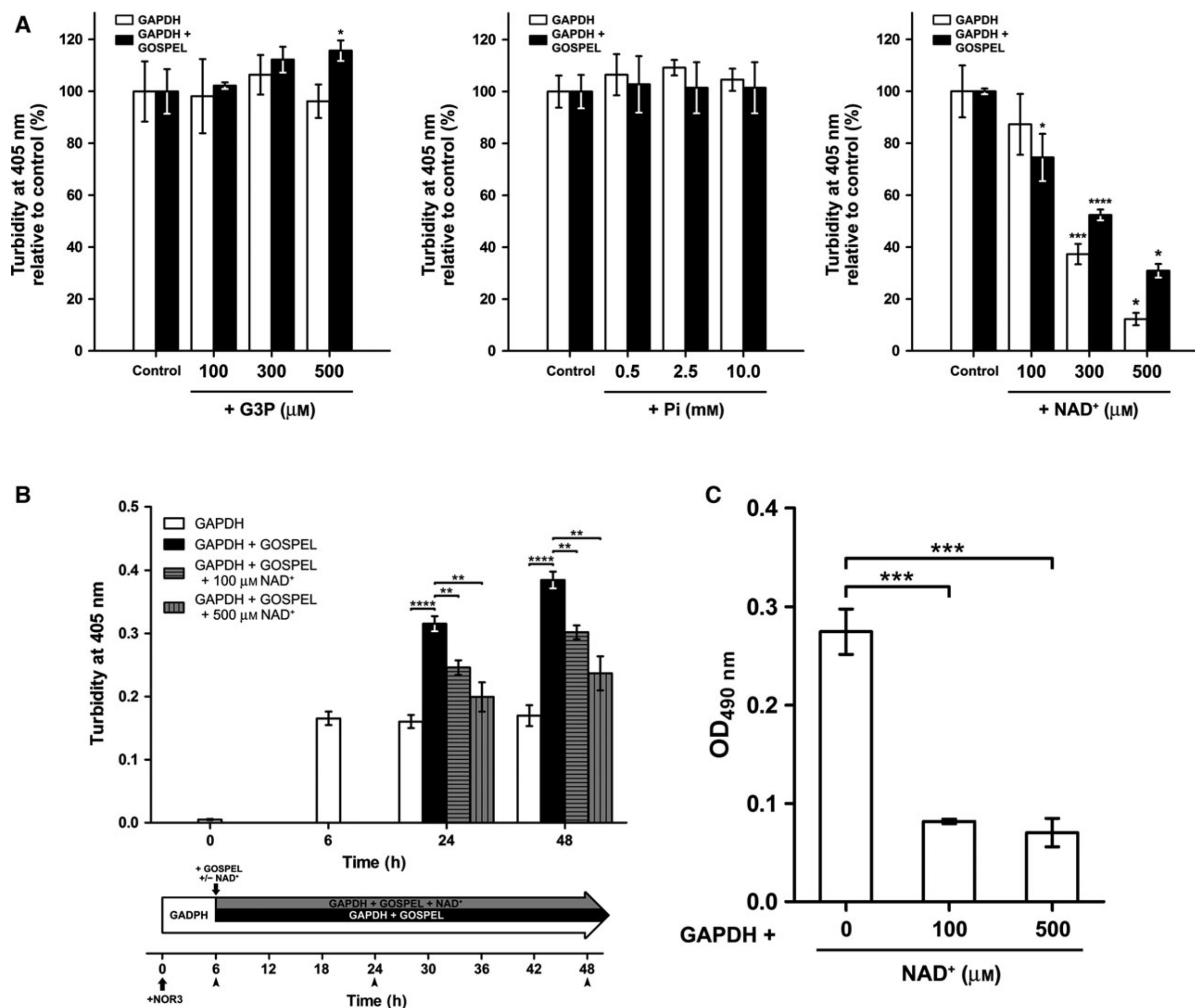


Fig. 6. Effect of NAD+, G3P and Pi on the NOR3 induced GOSPEL-free and GOSPEL-containing GAPDH aggregation. (A) GAPDH and GAPDH-GOSPEL reaction mixtures were incubated for 30 min with the indicated concentration of G3P, Pi and NAD+ before the addition of NOR3. The turbidity of each sample was measured after 24-h incubation with the NO donor. (B) After 6-h incubation, the GAPDH mixture was divided into aliquots that were subsequently incubated with GOSPEL alone or in the presence of the indicated amount of NAD+. Turbidity was measured at 0, 6, 24, and 48 h. (C) GAPDH-GOSPEL interaction in the absence or presence of NAD+ was analyzed by ELISA as described in Materials and methods. Bars represent the mean ± SD of triplicate determinations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ vs the corresponding control.

than GAPDH, could have a protective role by recruiting oxidized proteins into the aggregates to facilitate their removal by the proteasomal system or autophagy [28,29]. In this context, special consideration should be given to the potential protective function bestowed by NAD+ on the NO-induced GAPDH aggregation.

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Author contributions

JAC and MEC planned the experiments; MCG, JMR, MCI, CJMS, and MEC performed the experiments; JAC and MEC wrote the paper.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Gel filtration chromatography of GAPDH.

Fig. S2. Effect of NOR3 on GAPDH activity.

Fig. S3. Crystal structure of human GAPDH.