Compensatory role of Neuroglobin in nervous and non-nervous cancer cells in response to the nutrient deprivation

Marco Fiocchetti *, Manuela Cipolletti, Maria Marino *
Department of Science, University of Roma Tre, Viale Guglielmo Marconi, Roma, Italy

* maria.marino@uniroma3.it (MM); marco.fiocchetti@uniroma3.it (MF)

Abstract

Environmental factors or adverse growth conditions that may reduce cell function or viability are considered stress. The cell ability to sense and respond to environmental stresses determine its function and survival destiny. We recently defined Neuroglobin (NGB), a heme-protein, as a compensatory protein in the 17β-Estradiol (E2) anti-apoptotic activity and as a sensor of oxidative stress in both neurons and breast cancer cells. Here, the possibility that NGB levels could represent a pivotal regulator of integrated response of cancer cells to stress has been evaluated. Data obtained in neuroblastoma and in breast cancer cell lines evidence that nutrient deprivation significantly up-regulated NGB levels at different time points. However, the analysis of autophagy activation led to exclude any possible role of stress- or E2-induced NGB in the upstream regulation of general autophagy. However, the over-expression of Flag-NGB in ERα stable transfected HEK-293 cells completely affects nutrient deprivation-induced decrease in cell number. In addition, reported results indicate that modulation of the anti-apoptotic Bcl-2 level may play a key role in the protective NGB function against energetic stress. Overall, these data define a role of NGB as compensatory protein in the cell machinery activated in response to stress and as general stress adaptation marker of cancer cells susceptible to oxidative stress, oxygen and, as demonstrated here for the first time, even to nutrient willingness. Despite the lacking of any direct NGB role on autophagic flux activated by energetic stress, NGB upregulation appears functional in delaying stress-related cell death allowing an appropriate cell response and adaptation to the changing extracellular conditions.

Introduction

During their life, cells may encounter unfavorable environmental conditions, which beyond a certain threshold became “stressors” activating the so-called stress response pathway, which, in turn, attempt to reduce cell damage and to maintain or re-establish cell homeostasis, or eventually eliminate damaged cells [1,2]. Stressor injury, like nutrient deprivation, hypoxia and oxidative stress, frequently occurs in living cells under either physiological or pathological states such as fasting, ischemia or solid tumor development [3].
In particular, cells triggered diverse strategies to cope with the fluctuation of nutrient availability including mobilization of stored (macro) molecules, recycling of cell components, and an overall reduction of functions [3]. Autophagy (macro-autophagy), an intracellular degradation pathway that occurs at basal levels in all cells during nutrient rich conditions, is one of the key cellular response upregulated in response to the nutrient withdrawal [4,5]. This process provides the cell with nutrients and energy by degrading cell components, by reducing the nutrient requirement, and decline of general functions; thus, autophagy allows cells to adapt themselves and function properly and coherently within the new environment. The failure of these strategies result in cells inability to respond properly and efficiently to stresses driving them to the apoptotic or necrotic death [3]. Pathological conditions, like solid cancer growth, conversely, are mainly linked to cells full adaption to the critical condition and escaping from the extracellular controls [6,7].

Neuroglobin (NGB) is an intracellular heme-globin. Several findings have supported a neuroprotective role of overexpressed NGB against hypoxic/ischemic and oxidative stress-related insults in both \textit{in vitro} and \textit{in vivo} experiments [8–14]. NGB operates as a mediator of stress sensing and cellular response coupling, in neuron-derived cells [10,15–17]. This role implies both the protein activation and/or its upregulation and the consequent triggering of adaptive cells response [10]. More recently, independent studies indicate that NGB protein level is differently modulated by oxidative stress and hypoxia in diverse extra nervous cancer cell lines and tissues [18,19]. In addition, we recently found NGB as a compensatory protein in the 17β-Estradiol (E2) activated pathway devoted to cell survival in both neuroblastoma (SK-N-BE) and primary neuron cells [8,20,21] as well as in extra nervous cancer cells [22–24]. Remarkably, as for neuron-derived cells, we demonstrated that NGB is a stress-inducible protein in breast cancer lines being upregulated in response to the oxidative stress, although low levels of O$_2$ are unable to impact on the NGB expression [23]. Altogether, these results suggest that NGB exerts a pivotal role in sensing extracellular stimuli/stresses and in transducing information within the cells to mount an appropriate cellular response in both nervous and non-nervous cells. However, if NGB could play any role in the cell response to low nutrient availability, particularly regarding on the regulation of autophagic flux, is still unknown.

Here, the effect of nutrient deprivation condition on NGB expression and its impact on the downstream activated cellular response mechanisms, have been evaluated in neuroblastoma cells (SK-N-BE), breast cancer cells (MCF-7) and human embryonic kidney cells (HEK-293), cellular models sensitive to E2, which will be used as positive control on NGB levels and functions.

**Material and methods**

**Reagents**

E2, Pen-Strep solution, RPMI-1640 media without phenol red, Dulbecco’s modified Eagle medium (DMEM) without phenol red, Earle’s Balanced Salt Solution (EBSS), charcoal-stripped fetal calf serum, protease inhibitor cocktail, bovine serum albumin fraction V (BSA), Bafilomycin A1, anti-Tubulin and anti-LC3 antibodies and G418 (Geneticin) selection antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bradford protein assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Anti-NGB, anti-Bcl2 and anti-p62 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The chemiluminescence reagent for Western blot super power ECL was obtained from Bio-Rad (Milan, Italy). All the other products were from Sigma-Aldrich. Analytical or reagent grade products were used without further purification.
Cell culture

SK-N-BE and MCF-7 cell lines (ATCC, LGC Standards S.r.l., Milan, Italy) were used at passage 4–8 and were grown in air containing 5% CO2 in phenol red free, RPMI-1640 or DMEM medium, respectively, containing 10% (v/v) charcoal-stripped fetal bovine serum, L-glutamine (2.0 mM), Pen-Strep solution (penicillin 100 U/ml and streptomycin (100 mg/ml) as previously described [8,24] (Control Medium). Nutrient deprivation condition was obtained by culturing cells in amino acid and serum free, Earle’s Balanced Salt Solution (EBSS, Sigma Aldrich) containing 1 g/L of D-glucose for the indicated times. Stable ERα-transfected HEK-293 (ERα-HEK-293) cell lines were routinely grown in media containing G418 50 mM [25]. Cell line authentication were periodically performed by amplification of multiple STR loci by BMR Genomics srl (Padua, Italy). Cells were simultaneously treated with the vehicle used to dissolve all drugs (ethanol/PBS 1:10, v/v), and/or E2 (1 or 10 nM), and/or Bafalomycin-A1 (Baf-A1, 100 nM). When indicated, E2 or Baf-A1 pretreatment were performed adding the compounds 1 h before. For nutrient deprivation, MCF-7, SK-N-BE or ERα-HEK-293, were cultured as above reported, washed 3 times with PBS then cultured in EBSS for the indicated time points.

Flag-NGB plasmid and cell transfection

The pcDNA-flag-NGB (Flag-NGB) was obtained by subcloning the NGB ORF from the NGBN1-pEGFP plasmid40 into the pcDNA-flag 3.1C. HEK-293 cells were grown to ~70% confluence and then transfected with pcDNA-flag-NGB plasmid using lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Four hours after transfection, the medium was changed and 24 h after the cells were treated as previously described.

Western blot assay

Protein extraction and Western blot assay were performed as previously reported [21]. Briefly, after treatment, cells were lysed and solubilized in the YY buffer (50 mM HEPES at pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA) containing 0.70% (w/v) SDS. Total proteins were quantified using the Bradford Protein Assay. Solubilized proteins (20 μg) were resolved by 10% or 15% SDS-PAGE at 100 V for 1 h at 24.0˚C and then transferred to nitrocellulose with the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA) for 10 or 7 min, respectively. The nitrocellulose was treated with filtered 5% (w/v) BSA in 138.0 mM NaCl, 25.0 mM Tris, pH 8.0, at 24.0˚C for 1 h and then probed overnight at 4.0˚C with either anti-NGB (final dilution 1:1000), anti-LC3 (final dilution 1:1000), anti-p62 (final dilution 1:1000), anti Bcl-2 (final dilution 1:1000), and anti-Tubulin (final dilution 1:1000) antibodies. The antibody reaction was visualized with the chemiluminescence Western blotting detection reagent (Amersham Biosciences, Little Chalfont, UK). The densitometric analyses were performed by ImageJ software for Microsoft Windows (National Institutes of Health, Bethesda, MD, USA).

Cell viability

ERα-HEK-293 cell lines transfected or not with Flag-NGB were grown to 70% confluence in 6-well plates and cultured with EBSS for the indicated time point. After treatment, cells were harvested with trypsin, and counted with Beckman Coulter Model ZM electronic particle (Palo Alto, Calif., USA).
Statistical analysis

The statistical analysis was performed by Student’s t-test with the INSTAT software system for Windows. In all cases, \( p < 0.05 \) was considered significant.

Results

Effect of nutrient deprivation on NGB levels

The effects of nutrient deprivation on the expression of NGB levels has been evaluated in human neuroblastoma cells (SK-N-BE) [8], breast cancer cells (MCF-7) [24] and the ER devoid HEK-293 [26] stable transfected with ER\( \alpha \) plasmid (ER\( \alpha \)-HEK-293). Cells were cultured in control medium or EBSS for 2, 4 and 6 h (Fig 1). E2 treatment (10 nM in MCF-7 and ER\( \alpha \)-HEK-293; 1 nM in SK-N-BE cells) has been used as positive control due to its well-known ability to upregulate NGB levels in these cell lines [8,22–24]. EBSS culturing increases NGB expression in all of the cell models considered (Fig 1A, 1B and 1C). Indeed, nutrient deprivation rapidly increases NGB level in SK-N-BE and MCF-7 cells (2h after the changing condition) while, in ER\( \alpha \)-HEK-293 cells EBSS treatment enhances NGB expression just 6h after cell exposure, supporting a cell-based difference on stress response. In order to assess possible synergistic effects between E2 treatment and nutrient deprivation condition, cells were pre-treated with E2 (10 nM in MCF7 and ER\( \alpha \)-HEK-293; 1 nM in SK-N-BE cells) 1h before the incubation with EBSS. In ER\( \alpha \)-HEK-293, E2 shorten the time necessary to increase NGB levels (Fig 1C). However, E2 pre-treatment does not enhance EBSS-induced NGB upregulation in any of the considered cell lines (Fig 1).

![Fig 1. Nutrient deprivation impact on NGB expression levels.](https://doi.org/10.1371/journal.pone.0189179.g001)
Nutrient deprivation and E2 effects on autophagic flux

Due to the strict relation between nutrient deprivation and autophagy [4,5], we next evaluated the activation of such cellular event in our cellular models at different time of nutrient deprivation exposure. Autophagy induction was evaluated by following the level of microtubule associated protein light chain 3 (LC3I) protein and its lipidated form (LC3II), which is the marker of the autophagosome number being accumulated on the autophagosome membrane where it remains until a complete degradation [27,28]. In all considered cell models, the amount of LC3II protein increases already 2 h after the exposure to nutrient deprivation suggesting a rapid cell response to the lack of nutrient availability. However, the accumulation of LC3II protein and, consequently, of autophagosomes, could be related to an effective increase of autophagy process and/or to a defect in the autophagolysosome formation associated with autophagy inhibition [27,28]. To solve this ambiguity, we analyzed the expression levels of p62 protein, also known as sequestrosome (SQSTM1), an autophagy cargo molecule that drive selected soluble molecules to the auto-phagolysosome for their degradation. Thus, p62 is considered as both an autophagy substrate and a marker of the autophagic flux being degraded when autophagy flux is allowed and accumulated when autophagy is impaired [27]. Fig 2 shows that the nutrient deprivation significantly leads to a rapid degradation of p62 protein parallel with the accumulation of LC3II protein sustaining an effective induction of autophagy flux induced by the EBSS culturing medium in selected cell lines. The cell treatment with the well-known autophagy inhibitor Baf-A1 (100 nM) [27] alone or 1h before the EBSS culturing resulted in a further LC3II accumulation (Fig 2). This additive effect indicates that nutrient deprivation-induced autophagosomes accumulation depends on the activation of autophagic flux not to its block.

In order to evaluate if the other NGB inducer (i.e., E2) (see Fig 1) modulates the autophagic flux, E2 effects were assessed. As shown in Fig 3, E2 (10 nM) stimulation leads to the accumulation of p62 protein 2h after the hormone stimulation in both MCF-7 and in ERα-HEK-293 cells, without any significant effect on the expression of LC3II (Fig 3A, 3A’, 3C and 3C’). Such effect appears to be rapid and transient in MCF-7 where it vanishes 4h after E2 stimulation (Fig 3A’), whereas it persists until 6h in ERα-HEK-293 (Fig 3C’), probably due to the ectopic expression of ERα in these latter cells. On the other hand, in neuroblastoma SK-N-BE cells, E2 (1nM) treatment enhances the amount of LC3II protein since 4h after the stimulation (Fig 3B) without a parallel reduction of p62 protein (Fig 3B’), indicating that E2 leads to the accumulation of autophagosome without any completion of the autophagic flux. Altogether, obtained data indicate that E2 could affect autophagy flux exerting an inhibitory role on it. Notably, in all cells considered the Baf-A1 (100 nM) pre-treatment 1 h before E2 stimulation does not result in a further increase of LC3II and/or p62 protein levels respect to what observed with the Baf-A1 treatment alone (Fig 3). The absence of any additive effects stimulating cells with both E2 and Baf-A1 suggests a possible common mechanism shared by the hormone and the autophagy inhibitor in the last stage of autophagy flux.

Involvement of NGB overexpression on the autophagic flux and cell viability during nutrient deprivation

Data reported in Figs 1 and 2 indicate that the EBSS-dependent up-regulation of NGB is not parallel with the induction of autophagy flux in ERα-HEK-293 cells where NGB increases after 6h, while autophagy is activated already 2h after. Such data lead to hypothesize that NGB induction is not directly linked to the autophagic flux activation. Therefore, in order to test the possible role of high levels of NGB in the autophagic process, ERα-HEK-293 cells were transiently transfected with pcDNA-Flag-NGB plasmid (Fig 4A). Both control and Flag-NGB
overexpressing cells were exposed to nutrient deprivation condition for 2, 4 or 6h. Notably, the levels of both autophagy markers (i.e., LC3II and p62) do not show any significant difference between not–transfected and Flag-NGB transfected cells (Fig 4B and 4C). Furthermore, even the very high NGB levels reached by its ectopic expression 6h after nutrient deprivation does not change the level of the autophagy flux with the respect to the not-transfected cells supporting that a possible direct effect of NGB levels on autophagy activation, could be ruled out. Prolonged/chronic exposure to nutrient deprivation stress could lead to apoptotic or necrotic cell death [3]. This evidence prompted us to verify if Flag-NGB overexpression could affect cell viability during short and prolonged exposure to nutrient deprivation condition. Fig 4D shows that EBBS culturing significantly reduced cell number 6h after the treatment, which is parallel with the increase of NGB levels in this cell line (See Fig 1C). Such decline in cell number is further enhanced 24 h after exposure to nutrient deprivation condition. Remarkably, in Flag-

https://doi.org/10.1371/journal.pone.0189179.g002
NGB cells, EBSS-dependent decrease in cell number after 6 and 24h exposure is completely abolished (Fig 4D). Evidence have indicated that death induced by nutrient deprivation is commonly mediated by mitochondrial apoptotic pathway that mainly involves several members of Bcl-2 family, including the anti-apoptotic protein Bcl-2 [3]. This led us to evaluate the effect of overexpressed NGB on Bcl-2 expression. As reported in Fig 4E, in Flag-NGB ERα-HEK-293 cells the expression of Bcl-2 results significantly increased, with respect to the not-transfected counterpart, in both vehicle condition and until 4h after the exposure to nutrient deprivation. On the other hand, the Bcl-2 protein levels in Flag-NGB cells does not result significantly different from what observed in not-transfected cells after 6h nutrient deprivation exposure, or even reduced after 24h of low nutrient availability. Overall, these results show that ectopic expression of NGB increases cell survival during a prolonged exposure to nutrient deprivation.

Fig 3. The effect of E2 on autophagy process. MCF-7 (A, A'), SK-N-BE (B, B') and ERα-HEK-293 (C, C') were treated with E2 (10nM in MCF-7 and ERα-HEK-293 and 1 nM in SK-N-BE) in presence or absence of Baf-A1 (100 nM; 1 h before) pretreatment, or with Baf-A1 (100 nM) alone at indicated time points. LC3 (A, B, and C) and p62 (A', B', C') protein expression were analyzed via western blot. The amount of protein was normalized on tubulin levels. For LC3 quantitation, the formula LC3II/(LC3 I + LC3II) has been applied. Top panels are representative western blots of three independent experiments. Bottom panels show results of densitometric analysis. Data are means ± SD of three different experiments. P<0.05 was determined with Student t-test vs Veh (*), E2 treated samples (˚) and Baf-A1 6h (§) sample.

https://doi.org/10.1371/journal.pone.0189179.g003
Neuroglobin compensatory role in cell response to nutrient deprivation

A

Flag

Tub

Veh 2 4 6 Veh 2 4 6

EBSS EBSS

CONTROL Flag- Ngb plasmid

B

LC3I

LC3II

Tub

Veh 2 4 6 Veh 2 4 6

EBSS EBSS

CONTROL Flag- Ngb plasmid

C

p62

Tub

Veh 2 4 6 Veh 2 4 6

EBSS EBSS

CONTROL Flag- Ngb plasmid

D

LC3II/LC3I+LC3II/Tubulin levels

Arbitrary units

Veh 2 4 6 Veh 2 4 6

EBSS EBSS

CONTROL Flag- Ngb plasmid

E

Bcl-2

Tub

Veh 2 4 6 24 Veh 2 4 6 24

EBSS EBSS

CONTROL Flag- Ngb plasmid

Bcl-2/Tubulin protein level (arbitrary units)

Veh 2 4 6 24 Veh 2 4 6 24

EBSS EBSS

CONTROL Flag- Ngb plasmid

* * * * * * * * * * * *
deprivation, and it is linked to a parallel-enhanced expression of the anti-apoptotic protein Bcl-2.

Discussion

NGB is a monomeric intracellular heme-globin, which attracted research interest in the last almost two decades because of its wide distribution in the brain and, in particular, of its well-known pro-survival effects against several type of extracellular insults when overexpressed [8–14]. In neuron derived cells and in extra nervous cancers, NGB expression is closely related and/or induced by stress conditions themselves like as hypoxia [29,30], oxidative stress (H$_2$O$_2$) [21,23,24], oxygen and glucose deprivation [31], and lipopolysaccharide treatment [20]. In addition, NGB ability to change its structure, reactivity, and function in response to intracellular redox state changes has further reinforced the idea that NGB, as a stress responsive-sensor, transfers the stress condition to the signal transduction pathways important for cell response to stress [17]. This evidence prompted us to evaluate the possible NGB modulation and function in the cell response to low nutrient availability. Indeed, although many research studies were aimed at evaluating the effect in neurons of ischemic injury and or oxygen and glucose deprivation on NGB expression [31–33], the role of nutrient stress on NGB protein levels is still unknown. Results reported here clearly indicate that culturing cells in starved condition positively modulates NGB protein levels in both neuroblastoma and breast cancer cell lines. In addition, such effects appear to share the same intracellular pathway activated by one of the main NGB inducer, E2, which does not exert any synergistic effects when given before the nutrient deprivation condition. Consistent with this, we recently prove that, at least in breast cancer cell line, both E2 and oxidative stress inducing compound Lead Acetate, led to the NGB expression through the activation of AKT pathway [34] (Fiocchetti et al. personal communication). It is possible that similar common mechanisms could be activated by nutrient deprivation to modulate NGB levels.

Nutrient withdrawal represents one of the main challenge that fast-growing cancer cells could encounter during cancer development [4]. The ability of cancer cells to adapt to stresses and to escape from cell death is fundamental for tumor growth and survival [4].

Autophagy is the key cellular responses that is promptly activated in response to nutrient stress [1,3–5]. It represents an homoestatic process based on the production of double membrane vesicle, autophagosomes, that expand to engulf citoplasmic components to degrade and use them as structure but also as an energy reserve [3,35,36]. Basal autophagy maintains

---

https://doi.org/10.1371/journal.pone.0189179.g004
protein and organelle quality control [36] and its rate is further enhanced during stressing condition to clear damaged organelles and recycle nutrients [3,35,36].

Accordingly, with literature, nutrient deprivation treatment leads to a rapid increase in the accumulation of the autophagosome marker LC3II with a parallel degradation of the p62 autophagy substrate in both neuroblastoma and breast cancer cells. Consistent with an effective increase in autophagy flux, by treating cells with autophagy inhibitor Baf-A1, a further increase in auto-phagosome accumulation during nutrient withdrawal occurs. On the contrary, E2 treatment blocks the continuation of autophagic flux in the cell model used. Despite of controversial evidence about the role of E2 on autophagy, showing the hormone ability to both induce [37,38] or block [39–41] autophagic flux, our data sustain the role of E2 as an anabolic hormone that, like as it occurs for insulin treatment [42], is expected to function suppressing the conserved catabolic process of self-digestion. Moreover, the absence of any synergistic effects between E2 and Baf-A1 suggests that E2 and Baf-A1 share the same mechanisms in preventing the autophagic flux.

Differences between E2 and EBSS effects on autophagy lead to the paradoxical circumstance for which two different inducers of NGB overexpression oppositely affect the same intracellular mechanism. However, a possible direct involvement of up-regulated NGB in the general autophagy process could be kept out. Indeed, nutrient deprivation shows a different timing in the activation of autophagy process and the up-regulation of NGB protein levels, which sustains the lack of any direct relationship between such events. This is further confirmed by data demonstrating as the ectopic expression of NGB in ERα-HEK-293 cells does not change the autophagic flux markers (LC3II, p62) either in basal condition or after exposure to nutrient deprivation at those time points when the cellular response to nutrient withdrawal does not affect the NGB protein levels. Altogether, such evidence is consistent with other reported findings, which show that NGB overexpression does not exert any significant effect on the mRNA levels of upstream autophagy regulators Atg5, Atg7 and Beclin-1 [43].

Autophagy has been considered for long time a crucial process able to promote cancer survival under metabolic and genotoxic stresses which allows cancer resistance to treatment [4,35]. However, a mutually opposed survival and death-promoting role for autophagy has been suggested and mechanisms regulating such functions, in particular in cancer cells, are still far from resolution [4,35,36]. Remarkably, the E2 inhibitory effects on autophagy (present data) and its well-known anti-apoptotic functions in neuroblastoma [8] and breast cancer [24] cells, sustain the complexity of interrelationship between autophagy rate and cell death regulation.

As a whole, the lacking of any involvement of NGB overexpression in the autophagy activation does not rule out the possible role of stress upregulated NGB in the pro-survival mechanism activated by cells in response to extracellular nutrient deprivation. Indeed, an appropriate cell response to nutrient shortage is not limited to the autophagy process, being generally pointed to attempt cell survival waiting for “better times” [3]. Accordingly, here we found that NGB overexpression preserves cell viability after prolonged exposure to nutrient withdrawal further sustaining the pro-survival role of high levels of NGB during different type of insults [8–10,24,44,45]. Nutrient deprivation-induced cell death mainly occurs through the activation of intrinsic or mitochondrial apoptotic pathway [3]. Such process relies on the balance between pro-apoptotic (i.e. Bim, Bax, Bid) and anti-apoptotic (i.e. Bcl-2, Bcl-xL, Bcl-x) [46] members of Bcl-2 protein family. As elsewhere reported [47], glucose withdrawal-induced death is activated via the mitochondrial translocation of Bax and, in MCF-7 cells, it can be inhibited by Bcl-2 overexpression [47]. Such evidence confirms that the increased expression of Bcl-2 protein is tightly linked to pro-survival and anti-apoptotic events. Furthermore, NGB expression is related to a decreased or increased expression of pro-apoptotic or anti-apoptotic Bcl-2 members, respectively [21,48–51]. In addition,
E2-dependent up-regulation of NGB results pivotal in the hormone induced overexpression of Bcl-2 protein in breast cancer cell line [24]. Present reported findings show an increased level of Bcl-2 protein parallel with NGB overexpression. Therefore, although NGB may affect cell survival impinging on different pathways [17,21,51,52], the regulation of the Bcl-2 protein network may play a key role in the protective NGB function during energetic stress. In parallel, lowest Bcl-2 levels in NGB-overexpressed cells after a longer time of exposure to nutrient deprivation lead to hypothesize that the high levels of NGB could contrast cell death in a limited “time window” at the beginning of energetic stress-related insult. Indeed, stress-dependent induction of NGB could be functional to prevent accidental apoptosis during the exposure to low and shortened stress condition [52–55]. On the opposite, we recently proved as the E2-dependent up-regulation and mitochondrial re-localization of NGB is required to confer cell protection against high levels of oxidative stress [23,24].

A great amount of intracellular functions has been ascribed to overexpressed NGB, mainly linked to the well-known ability of the globin to exert pro-survival function [56–59]. Among the evidence put forward to define mechanisms underlying the protective function of NGB, several reports support a role of NGB in intracellular signaling impacting on metabolic, oxidative/hypoxia and survival/apoptotic pathways. NGB has been found upregulated by ischemia/hypoxia in cultured cell lines and primary mouse cortical neurons [10,33,29] and by oxidative stress [21] in neuroblastoma cell. In addition, NGB function as oxygen [15,60] and oxidative stress sensor [17] has been demonstrated. Overall, these data define a role of NGB as compensatory protein in the cell machinery activated in response to stress and as general stress adaptation marker of cancer cells susceptible to oxidative stress, oxygen and, as demonstrated here for the first time, even to nutrient willingness. Despite the lacking of any direct NGB role on autophagy flux activated by energetic stress, NGB upregulation appears functional in delaying stress-related cell death allowing an appropriate cell response and adaptation to the changing extracellular conditions. Therefore, NGB could represent a link between the cell ability to sense nutrient withdrawal, and the impairment of cell death during acute stress phase linked to the up-regulation of Bcl-2 anti-apoptotic protein.

Modulation of endogenous cellular defense mechanisms activated in response to stress represents an innovative approach in therapy in diseases causing chronic tissue damage, like as cancer [7]. Here reported observation add to our growing knowledge of the importance of NGB in mechanisms and structures involved in cellular stress response opening novel avenue in the development of therapeutic interventions.

Acknowledgments
This work was supported by grant from Associazione Italiana Ricerca sul Cancro (AIRC, IG#15221) to M.M.

Author Contributions
Conceptualization: Marco Fiocchetti, Maria Marino.
Data curation: Marco Fiocchetti, Maria Marino.
Funding acquisition: Maria Marino.
Investigation: Marco Fiocchetti, Manuela Cipolletti.
Methodology: Manuela Cipolletti.
Supervision: Maria Marino.
Writing – original draft: Marco Fiocchetti.
References
1. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. Int J Cell Biol. 2010; 2010: 214074. https://doi.org/10.1155/2010/214074 PMID: 20182529
2. Simmons SO, Fan CY, Ramabhadran R. Cellular stress response pathway system as a sentinel ensemble in toxicological screening. Toxicol Sci. 2009; 111: 202–225. https://doi.org/10.1093/toxsci/kfp140 PMID: 19957893
3. Caro-Maldonado A, Muñoz-Pinedo C. Dying for Something to Eat: How Cells Respond to Starvation. Open Cell Signal J. 2011; 3: 42–51.
4. Jain K, Paranandi KS, Basu A. Autophagy in breast cancer and its implications for therapy. Am J Cancer Res. 2013; 3: 251–265. PMID: 23841025
5. Russell RC, Yuan HX, Guan KL. Autophagy regulation by nutrient signaling. Cell Res. 2014; 24: 42–57. https://doi.org/10.1038/cr.2013.166 PMID: 24343578
6. Calabrese V, Cornelius C, Dinkova-Kostova AT, Calabrese EJ, Mattson MP. Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders. Antioxid Redox Signal. 2010; 13: 1763–1811. https://doi.org/10.1089/ars.2009.3074 PMID: 20446769
7. Chircop M, Speidel D. Cellular stress responses in cancer and cancer therapy. Front Oncol. 2014; 4: 304. https://doi.org/10.3389/fonc.2014.00304 PMID: 25401089
8. De Marinis E, Ascenzi P, Pellegrini M, Galluzzo P, Bultzomi P, Arevalo MA, et al. 17β-estradiol-a new modulator of neuroglobin levels in neurons: role in neuroprotection against H2O2-induced toxicity. Neuronalig. 2010; 18: 223–235.
9. Fordel E, Thijs L, Martinet W, Lenjou M, Laufs T, Van Bockstaele D, et al. Neuroglobin and cytoglobin overexpression protects human SH-SY5Y neuroblastoma cells against oxidative stress-induced cell death. Neurosci Lett. 2006; 410: 146–151. https://doi.org/10.1016/j.neulet.2006.09.027 PMID: 17095159
10. Greenberg DA, Jin K, Khan AA. Neuroglobin: an endogenous neuroprotectant. Curr Opin Pharmacol. 2008; 8: 20–24. https://doi.org/10.1016/j.coph.2007.09.003 PMID: 17942367
11. Khan AA, Mao XO, Banwait S, Jin K, Greenberg DA. Neuroglobin attenuates beta-amyloid neurotoxicity in vitro and transgenic Alzheimer phenotype in vivo. Proc Natl Acad Sci U S A. 2007; 104: 19114–19119. https://doi.org/10.1073/pnas.0706167104 PMID: 18025470
12. Khan AA, Wang Y, Sun Y, Mao XO, Xie L, Miles E, et al. Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. Proc Natl Acad Sci U S A. 2006; 103: 17944–17948. https://doi.org/10.1073/pnas.0607497103 PMID: 17098866
13. Sun Y, Jin K, Peet A, Mao XO, Xie L, Greenberg DA. Neuroglobin protects the brain from experimental stroke in vivo. Proc Natl Acad Sci U S A 2003; 100: 3497–3500. https://doi.org/10.1073/pnas.0637726100 PMID: 12621155
14. Yu Z, Liu N, Liu J, Yang K, Wang X. Neuroglobin, a Novel Target for Endogenous Neuroprotection against Stroke and Neurodegenerative Disorders. Int J Mol Sci. 2012; 13: 6995–7014. https://doi.org/10.3390/ijms13066995 PMID: 22837676
15. Khan AA, Mao XO, Banwait S, DerMarderosian CM, Bokoch GM, Jin K, et al. Regulation of hypoxic neuronal death signaling by neuroglobin. FASEB J. 2008; 22: 1737–1747. https://doi.org/10.1096/fj.07-100784 PMID: 18198211
16. Sun Y, Jin K, Mao XO, Zhu Y, Greenberg DA. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. Proc Natl Acad Sci U S A. 2001; 98: 15306–15311. https://doi.org/10.1073/pnas.251466696 PMID: 11742077
17. Watanabe S, Takahashi N, Uchida H, Wakasugi K. Human neuroglobin functions as an oxidative stress-responsive sensor for neuroprotection. J Biol Chem. 2012; 287: 30128–30138. https://doi.org/10.1074/jbc.M112.373361 PMID: 22787149
18. Emara M, Saleh N, Allalunis-Turner J. Expression and hypoxic up-regulation of neuroglobin in human glioblastoma cells. Mol Oncol. 2009; 3: 45–53. https://doi.org/10.1016/j.molonc.2008.11.002 PMID: 19383366
19. Oleksiewicz U, Daskoulidou N, Liloglou T, Tasopoulos K, Bryan J, Gosney JR, et al. Neuroglobin and myoglobin in non-small cell lung cancer: expression, regulation and prognosis. Lung Cancer. 2011; 74: 411–418. https://doi.org/10.1016/j.lungcan.2011.05.001 PMID: 21640426
Neuroglobin compensatory role in cell response to nutrient deprivation

20. De Marinis E, Acaz-Fonseca E, Arevalo MA, Ascenzi P, Fiocchetti M, Marino M, et al. 17β-Oestradiol anti-inflammatory effects in primary astrocytes require oestrogen receptor β-mediated neuroglobin up-regulation. J Neuroendocrinol. 2013; 25: 260–270. https://doi.org/10.1111/jne.12007 PMID: 23190172

21. De Marinis E, Fiocchetti M, Acconia F, Ascenzi P, Marino M. Neuroglobin upregulation induced by 17β-estradiol sequesters cytochrome c in the mitochondria preventing H2O2-induced apoptosis of neuroblastoma cells. Cell Death Dis. 2013; 4: e508. https://doi.org/10.1038/cddis.2013.30 PMID: 23429294

22. Fiocchetti M, Cipolletti M, Leone S, Ascenzi P, Marino M. Neuroglobin overexpression induced by the 17β-Estradiol-Estrogen receptor-alpha Pathway reduces the sensitivity of MCF-7 Breast cancer cell to paclitaxel. IUBMB Life. 2016; 68: 645–651. https://doi.org/10.1002/iub.1522 PMID: 27312786

23. Fiocchetti M, Cipolletti M, Leone S, Naldini A, Carraro F, Giordano D, et al. Neuroglobin in Breast Cancer Cells: Effect of Hypoxia and Oxidative Stress on Protein Level, Localization, and Anti-Apoptotic Function. PLoS One. 2016; 11: e0154959. https://doi.org/10.1371/journal.pone.0154959 PMID: 27149623

24. Fiocchetti M, Nuzzo MT, Totta P, Acconia F, Ascenzi P, Marino M. Neuroglobin, a pro-survival player in estrogen receptor alpha-positive cancer cells. Cell Death Dis. 2014; 5: e1449. https://doi.org/10.1038/cddis.2014.418 PMID: 25299774

25. Pesiri V, Totta P, Segatto M, Bianchi F, Pallottini V, Marino M, et al. Estrogen receptor α L429 and A430 regulate 17β-estradiol-induced cell proliferation via CREB1. Cell Signal. 2015; 27: 2380–2388. https://doi.org/10.1016/j.cellsig.2015.08.021 PMID: 26348925

26. Shanie EK, Onitilo AA, Huang W, Kim K, Zang C, Engel JM, et al. Prognostic significance of full-length estrogen receptor β expression in stage I-III triple negative breast cancer. Am J Transl Res. 2015; 7: 1246–1259. PMID: 26328009

27. Kilionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2012; 8: 445–544. https://doi.org/10.4161/auto.19496 PMID: 22966490

28. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell. 2010; 140: 313–326. https://doi.org/10.1016/j.cell.2010.01.028 PMID: 20447757

29. Schmidt-Kastner R, Haberkamp M, Schmitz C, Hankeln T, Burmester T. Neuroglobin mRNA expression after transient global brain ischemia and prolonged hypoxia in cell culture. Brain Res. 2006; 1103: 124–133. https://doi.org/10.1016/j.brainres.2006.05.047 PMID: 16796995

30. Emara M, Turner AR, Allalunis-Turner J. Hypoxic regulation of cytoglobin and neuroglobin expression in human normal and tumor tissues. Cancer Cell Int. 2010; 10: 33. https://doi.org/10.1186/1475-2867-10-33 PMID: 20828399

31. Fordel E, Thijs L, Martinet W, Schrijvers D, Moens L, Dewilde S. Anoxia or oxygen and glucose deprivation in SH-SY5Y cells: a step closer to the unraveling of neuroglobin and cytoglobin functions. Gene. 2007; 398: 114–122. https://doi.org/10.1016/j.gene.2007.03.022 PMID: 17532579

32. Shang A, Zhou D, Wang L, Gao Y, Fan M, Wang X, et al. Increased neuroglobin levels in the cerebral cortex and serum after ischemia-reperfusion insults. Brain Res. 2006; 1078: 219–226. https://doi.org/10.1016/j.brainres.2006.01.064 PMID: 16492379

33. Yu Z, Liu N, Li Y, Xu J, Wang X. Neuroglobin overexpression inhibits oxygen-glucose deprivation-induced mitochondrial permeability transition pore opening in primary cultured mouse cortical neurons. Neurorobiol Dis. 2013; 56: 95–103. https://doi.org/10.1016/j.nbd.2013.04.015 PMID: 23639789

34. Fiocchetti M, Cipolletti M, Ascenzi P, Marino M. Dissecting the 17β-estradiol pathways necessary for Neuroglobin anti-apoptotic activity in breast cancer J Cell Physiol. 2017; In press.

35. Karantza-Wadsworth V, White E. Role of autophagy in breast cancer. Autophagy. 2007; 3: 610–613. PMID: 17786023

36. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nat Rev Cancer. 2007; 7: 961–967. https://doi.org/10.1038/nrc2254 PMID: 17972889

37. Fan D, Liu SY, van Hasselt CA, Vlantis AC, Ng EK, Chu R, et al. Estrogen receptor alpha induces prosurvival autophagy in papillary thyroid cancer via stimulating reactive oxygen species and extracellular signal regulated kinases. J Clin Endocrinol Metab. 2015; 100: E561–571. https://doi.org/10.1210/jc. 2014-3257 PMID: 25594859

38. Yang YH, Chen K, Li B, Chen JW, Zheng XF, Wang YR, et al. Estradiol inhibits osteoblast apoptosis via promotion of autophagy through the ER-ERK-mTOR pathway. Apoptosis. 2013; 18: 1363–1375. https://doi.org/10.1007/s10495-013-0867-x PMID: 23743762

39. Li L, Chen J, Sun S, Zhao J, Dong X, Wang J. Effects of Estradiol on Autophagy and Nrf-2/ARE Signals after Cerebral Ischemia. Cell Physio Biochem. 2017; 41: 2027–2036. https://doi.org/10.1159/ 000475433 PMID: 28419990
40. Mei J, Zhu XY, Jin LP, Duan ZL, Li DJ, Li MQ. Estrogen promotes the survival of human secretory phase endometrial stromal cells via CXCL12/CXCR4 up-regulation-mediated autophagy inhibition. Hum Reprod. 2015; 30: 1677–1689. https://doi.org/10.1093/humrep/dev100 PMID: 25976655

41. Totta P, Busonero C, Leone S, Marino M, Acconcia F. Dynamin II is required for 17β-estradiol signaling and autophagy-based ERα degradation. Sci Rep. 2016; 6: 23727. https://doi.org/10.1038/srep23727 PMID: 27009360

42. Kanazawa T, Taneike I, Akaishi R, Yoshizawa F, Furuya N, Fujimura S, et al. Amino acids and insulin control autophagic proteolysis through different signaling pathways in relation to mTOR in isolated rat hepatocytes. J Biol Chem. 2004; 279: 8452–8459. https://doi.org/10.1074/jbc.M306337200 PMID: 14610086

43. Deng SY, Ai YH, Gong H, Wu L, Chen CX, Wang YM, et al. Effect of neuroglobin on oxygen-glucose deprivation and reoxygenation induced autophagy in a human neuroblastoma cell line. Zhonghua Yi Xue Za Zhi. 2017; 97: 1505–1509. https://doi.org/10.3760/cma.j.issn.0376-2491.2017.19.016 PMID: 28536644

44. Antao ST, Duong TT, Aran R, Witting PK. Neuroglobin overexpression in cultured human neuronal cells protects against hydrogen peroxide insult via activating phosphoinositide-3 kinase and opening the mitochondrial K(ATP) channel. Antioxid Redox Signal. 2010; 13: 769–781. https://doi.org/10.1089/ars.2009.2977 PMID: 20362558

45. Zhou G, Shan P, Hu X, Zhang X, Zhou S. Neuroprotective effect of TAT PTD-Ngb fusion protein on primary cortical neurons against hypoxia-induced apoptosis. Neuronal Sci. 2013; 34: 1771–1778. https://doi.org/10.1007/s10471-013-0333-9 PMID: 23456422

46. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007; 35: 495–516. https://doi.org/10.1080/01926230701320337 PMID: 17562483

47. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB. Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol Cell Biol. 2001; 21: 5899–5912. https://doi.org/10.1128/MCB.21.17.5899-5912.2001 PMID: 11486029

48. Ascenzi P, di Masi A, Leboffe L, Fiocchetti M, Nuzzo MT, Brunori M, et al. Neuroglobin: From structure to function in health and disease. Mol Aspects Med. 2016; 52: 1–48. https://doi.org/10.1016/j.mam.2016.10.004 PMID: 27825818

49. Lan WB, Lin JH, Chen XW, Wu CY, Zhong GX, Zhang LQ, et al. Overexpressing neuroglobin improves functional recovery by inhibiting neuronal apoptosis after spinal cord injury. Brain Res. 2014; 1562: 100–108. https://doi.org/10.1016/j.brainres.2014.03.020 PMID: 24765030

50. Lin Y, Cai B, Xue XH, Fang L, Wu ZY, Wang N. TAT-mediated delivery of neuroglobin attenuates apoptosis induced by oxygen-glucose deprivation via the Jak2/Stat3 pathway in vitro. Neuronal Res. 2015; 37: 531–538. https://doi.org/10.1179/1743132814Y.0000000420 PMID: 25023896

51. Yu Z, Poppe JL, Wang X. Mitochondrial mechanisms of neuroglobin’s neuroprotection. Oxid Med Cell Longev. 2013; 2013: 756989. https://doi.org/10.1155/2013/756989 PMID: 23634236

52. Brittain T, Skommer J, Raychaudhuri S, Birch N. An antiapoptotic neuroprotective role for neuroglobin. Int J Mol Sci. 2011; 11: 2306–2321. https://doi.org/10.3390/ijms11062306 PMID: 20640154

53. Brittain T, Skommer J, Henty K, Birch N, Raychaudhuri S. A role for human neuroglobin in apoptosis. IUBMB Life. 2010; 62: 878–885. https://doi.org/10.1002/iub.405 PMID: 21190290

54. Raychaudhuri S, Skommer J, Henty K, Birch N, Brittain T. Neuroglobin protects nerve cells from apoptosis by inhibiting the intrinsic pathway of cell death. Apoptosis. 2010; 15: 401–411. https://doi.org/10.1007/s10495-009-0436-5 PMID: 20091232

55. Yang Y, Kitagaki J, Dai RM, Tsai YC, Lorick KL, Ludwig RL, et al. Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. Cancer Res. 2007; 67: 9472–9481. https://doi.org/10.1158/0008-5472.CAN-07-0568 PMID: 17909057

56. Burmester T, Hankeln T. What is the function of neuroglobin? J Exp Biol. 2009; 212: 1423–1428. https://doi.org/10.1242/jeb.000729 PMID: 19411534

57. Herold S, Fago A, Weber RE, Dewilde S, Moens L. Reactivity studies of the Fe(III) and Fe(II)NO forms of human neuroglobin reveal a potential role against oxidative stress. J Biol Chem. 2004; 279: 22841–22847. https://doi.org/10.1074/jbc.M313732200 PMID: 15020597

58. Jin K, Mao XO, Xie L, Khan AA, Greenberg DA. Neuroglobin protects against nitric oxide toxicity. Neurosci Lett. 2008; 430: 135–137. https://doi.org/10.1016/j.neulet.2007.10.031 PMID: 18035490

59. Nicolis S, Monzani E, Ciaccio C, Ascenzi P, Moens L, Casella L. Reactivity and endogenous modification by nitrite and hydrogen peroxide: does human neuroglobin act only as a scavenger? Biochem. 2007; J 407: 89–99.
60. Hota KB, Hota SK, Srivastava RB, Singh SB. Neuroglobin regulates hypoxic response of neuronal cells through Hif-1alpha- and Nrf2-mediated mechanism. J Cereb Blood Flow Metab. 2012; 32: 1046–1060. https://doi.org/10.1038/jcbfm.2012.21 PMID: 22472608