

Carbimazole is an inhibitor of protein synthesis and protects from neuronal hypoxic damage *in vitro*

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The abbreviations used are: Ac-DEVD-AMC, N-acetyl-(aspartic acid – glutamic acid – valine – aspartic acid) 7-amino-4-methylcoumarin; AMPK, cyclic adenosine monophosphate-dependent protein kinase; cAMP, cyclic adenosine monophosphate; CHX, cycloheximide; eEF2, eukaryotic elongation factor 2; eEF2K, eukaryotic elongation factor 2 kinase; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; LDH, lactate dehydrogenase; PKA, protein kinase A; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Abstract

Oxygen deprivation during ischemic or hemorrhagic stroke results in ATP-depletion, loss of ion homeostasis, membrane depolarisation, and excitotoxicity. Pharmacologic restoration of cellular energy supply may offer a promising concept to reduce hypoxic cell injury. In this study we investigated whether carbimazole, a thionamide used to treat hyperthyroidism, reduces neuronal cell damage in oxygen-deprived human SK-N-SH cells or primary cortical neurons. Our results revealed that carbimazole induces an inhibitory phosphorylation of eukaryotic elongation factor eEF2 that was associated with a marked inhibition of global protein synthesis. Translational inhibition resulted in significant bioenergetic savings, preserving intracellular ATP-content in oxygen-deprived neuronal cells and diminishing hypoxic cellular damage. Phosphorylation of eEF2 was mediated by AMP-activated protein kinase and eEF2 kinase. Carbimazole also induced a moderate calcium influx and a transient cyclic adenosine monophosphate increase. To test whether translational inhibition generally diminishes hypoxic cell damage when ATP-availability is limiting, the translational repressors cycloheximide and anisomycin were used. Cycloheximide and anisomycin also preserved ATP-content in hypoxic SK-N-SH cells and significantly reduced hypoxic neuronal cell damage. Taken together, these data support a causal relation between the pharmacologic inhibition of global protein synthesis and efficient protection of neurons from ischemic damage by preservation of high-energy metabolites in oxygen-deprived cells. Furthermore, our results indicate that carbimazole or other translational inhibitors may be interesting candidates for the development of new organ-protective compounds. Their chemical structure may be used for computer-assisted drug design or screening of compounds to find new agents with the potential to diminish neuronal damage under ATP-limited conditions.

Introduction

Ischemic or hemorrhagic stroke remain leading causes of death and disability worldwide (Barreto, 2011; Sahni and Weinberger, 2007). Current treatment strategies are primarily focused on the control of bleeding (Sahni and Weinberger, 2007), restoration of blood flow by thrombolytic therapies (Barreto, 2011), or lowering of intracranial pressure (Sahni and Weinberger, 2007) but are limited in success as they are aimed at the acute phase of brain damage and neglect that a significant amount of neuronal tissue damage develops progressively in the penumbra after the initial insult. Crucial molecular changes in penumbral tissue potentiate secondary brain injury, especially when ATP supply is insufficient to maintain ion homeostasis (Stankowski and Gupta, 2011; White *et al.*, 2000). Loss of high-energy phosphate compounds results in the failure of ion-motive ATPases, membrane depolarization, excitotoxic glutamate release, calcium overload, the induction of phospholipases and proteases, the formation of radicals and inflammatory mediators, and translation of damage-promoting proteins such as inducible nitric oxide synthase, cyclooxygenase-2, or matrix metalloproteinases (Cunningham *et al.*, 2005; Stankowski and Gupta, 2011; White *et al.*, 2000; Won *et al.*, 2002). There is a high correlation between the extent of neurological recovery and the volume of penumbra that escapes infarction (Furlan *et al.*, 1996); therefore, neuroprotective drugs with the potential to inhibit deleterious effects in the penumbra should improve clinical outcome.

Depression of protein translation, a dominant energy consuming cellular activity, results in substantial bioenergetic savings (Hand and Hardewig, 1996). Increased availability and reallocation of cellular energy to vitality-preserving mechanisms such as maintenance of ionic equilibrium or cellular repair may become critical for survival, especially under conditions of insufficient oxygen and nutrient supply (Boutilier and St Pierre, 2000; Wang *et al.*, 2008). Inhibition of protein synthesis during ischemia may also prevent the translational induction of mediators associated with nitration and oxidation of lipids, proteins and DNA, inhibition of

mitochondrial respiration, inflammation, increased intracranial pressure, or even hemorrhage (Cunningham *et al.*, 2005; Won *et al.*, 2002).

The majority of energy used in translation is consumed during elongation (Browne and Proud, 2002). In mammalian cells peptide-chain elongation requires eukaryotic elongation factor 2 (eEF2) that promotes the translocation of peptidyl-tRNA from the A site to the P site on the ribosome (Jorgensen *et al.*, 2006). Phosphorylation of eEF2 at Thr56 by eEF2 kinase (eEF2K) impairs interaction of eEF2 with the ribosome (Carlberg *et al.*, 1990) and is sufficient for the inhibition of mRNA translation (Ryazanov *et al.*, 1988). Enzymatic function of eEF2K is dependent upon calcium and calmodulin (Ryazanov, 1987), however, phosphorylation of eEF2K by cAMP-dependent protein kinase (PKA) (Redpath and Proud, 1993) or AMP-dependent protein kinase (AMPK) (Horman *et al.*, 2002) may lead to acquisition of calcium independent activity. Cytoprotective properties of enzymes involved in eEF2 regulation are well described. Activation of eEF2K promotes cell survival, reduces hypoxic injury, and regulates autophagy in response to nutrient deprivation (Py *et al.*, 2009; Terai *et al.*, 2005). AMPK preserves energy homeostasis (Hardie, 2004), attenuates ischemic cell damage (Zhang *et al.*, 2010), inflammation (Salminen *et al.*, 2011), arrhythmias (Wong *et al.*, 2009), hypertrophy (Chan *et al.*, 2004), structural remodeling (Du *et al.*, 2008), or plaque formation in Alzheimer's disease (Greco *et al.*, 2009). Additionally, it promotes neurogenesis, angiogenesis, and vascular function (Li and McCullough, 2010).

Carbimazole is a heterocyclic thiourylene used to treat hyperthyroidism. It prevents the thyroid peroxidase catalyzed iodination of L-tyrosine and thus reduces the production of the thyroid hormones T3 and T4. Amelioration of acute tissue damage by carbimazole in patients has not been investigated. However, *in vitro* studies have shown that carbimazole may inhibit inflammation (Humar *et al.*, 2008) and neuronal apoptosis (Humar *et al.*, 2009). Furthermore, structurally related thiourylenes have been implicated in the reduction of excitotoxicity (Zhan *et al.*, 1998), radical scavenging (Steen and Michenfelder, 1980), the induction of

cytoprotective proteins (Roesslein *et al.*, 2008), and may be used in brain injured patients to lower intracranial pressure and to improve cerebral oxygenation (Nordby and Nesbakken, 1984).

Because of the cytoprotective mechanisms attributed to heterocyclic thiourylenes it is plausible to analyze their therapeutic potential in ischemic neurons. Loss of high-energy phosphate compounds in ischemic neurons triggers a destructive biochemical cascade culminating in neuronal cell death. Therefore, the aim of the present study was to examine carbimazole-mediated effects on global protein synthesis, its impact on high-energy phosphate supply, and whether bioenergetic savings due to translational repression reduce neuronal damage following oxygen deprivation.

Materials and Methods

Cell culture and treatment

The human neuronal cell line SK-N-SH was purchased from the American Tissue Culture Collection and maintained in Eagle's minimum essential medium, supplemented with 1 mM sodium pyruvate, 2 mM Glutamax, 1500 mg/l sodium bicarbonate, 100 IU streptomycin, 100 IU penicillin, and 10% fetal calf serum (FCS). For culture of primary neurons, cortices of C57/BL6 neonates (P0-P2) were collected in cold modified Eagle's medium supplemented with 2 mM Glutamax, cut with scissors to smaller pieces, trypsinized (0.25% trypsin) for 10 min at 37°C, and dissociated in Hank's balanced salt solution containing 10 mM HEPES-KOH/pH 7.9, 3 mg/ml bovine serum albumin, 12 mM MgSO₄, 0.025% DNase I, and 0.4 mg/ml soybean trypsin inhibitor (Applicem, Darmstadt, Germany) as described previously (Lin et al., 2013). The dissociated cells were collected from the supernatant after centrifugation at 100 g for 5 min and neurons were enriched by a second centrifugation at 800 g for 5 min before they were seeded on Poly-d-Lysine coated tissue culture plates in Dulbecco's modified Eagle's medium containing 10% FCS and 2 mM Glutamax at 37°C with 8% CO₂. After 3 h, the medium was replaced by Neurobasal-A supplemented with 2% B27-supplement and 1 mM Glutamax. All materials used for cell culture were from Life Technologies (Carlsbad, CA) unless indicated otherwise.

Cells were treated with carbimazole (LTK Laboratories, St. Paul, MN), thapsigargin (Calbiochem, San Diego, CA), forskolin (Calbiochem), cAMPS-Rp (Tocris Bioscience, Bristol, UK), PKI 14-22 amide (Calbiochem), rapamycin (Calbiochem), dorsomorphin dihydrochloride (Calbiochem), camstatin (Tocris Bioscience), CGS 9343B (Tocris Bioscience), cycloheximide (Sigma, St. Louis, MO), or anisomycin (Calbiochem) as indicated. Oxygen-deprivation was induced by moving cell cultures to freshly prepared growth medium that has been pre-equilibrated in a hypoxic atmosphere for 15 h (5% CO₂,

95% N₂). To induce hypoxic cell death, cell culture was continued for 72 h in a controlled humidified atmosphere of 5% CO₂, 95% N₂ at 37°C.

Immunoblotting

Cells were lysed in 50 mM Tris-HCl/pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 10% glycerol, and 0.1% bromphenol blue in tissue culture plates. Lysates were sonicated, boiled for 5 min, and equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, blocked in 0.2% I-Block™ (Life Technologies), 50 mM Tris-HCl/pH 7.6, 150 mM NaCl, and 0.1% TWEEN 20, and incubated with antibodies raised against eEF2, phospho-eEF2(Thr56), AMPK, phospho-AMPK(Thr172; clone 40H9), pro-caspase 3, cleaved PARP, or α -tubulin according to the manufacturer's recommendations (Cell Signaling, Danvers, MA). Specific protein bands were visualized using horseradish-peroxidase conjugated anti-rabbit IgGs and enhanced chemiluminescence reagents (GE Healthcare, Munich, Germany).

Metabolic labeling

Cells were synchronized over night in 24 well plates using FCS free Eagle's minimum essential medium before treatment with translational inhibitors for 4 h. Then, cultures were washed two times for 20 min in methionine free Eagle's minimum essential medium (MP Biomedicals, Illkirch, France) to deplete internal methionine pools before cells were pulse labeled with 200 μ Ci/ml of [³⁵S]methionine (PerkinElmer, Rodgau, Germany) for 2 h and finally lysed in 50 mM Tris-HCl/pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 10% glycerol, and 0.1% bromphenol blue. Proteins were denatured by boiling for 5 min and separated by 10% SDS-PAGE before gels were fixed in 25% isopropanol and 10% acetic acid for 30 min, stained in 10% acetic acid containing 0.006% Coomassie G250 for 2 h, destained

in 10% acetic acid for 6 h, and dried on a Whatman 3MM paper for 2 h at 80°C. [³⁵S]methionine incorporation into proteins was visualized by x-ray autoradiography.

Measurement of intracellular calcium

Intracellular cytoplasmatic calcium measurements were performed by fura-2 labeling of SK-N-SH cells. Cultures were synchronized over night by starvation, treated with carbimazole or thapsigargin in calcium-free or calcium containing RPMI-medium (Genaxxon Bioscience, Ulm, Germany) for 1 h and loaded with 2 μM fura-2 acetoxymethyl ester (Molecular Probes, CA) for the last 30 min of the experiment at 37°C in the dark. Fluorescence was excited at 340 nm and emission was recorded at 510 nm by a spectrofluorophotometer (SpectraMax® GeminiXS, Molecular Devices, Sunnyvale, CA).

Measurement of intracellular cyclic adenosine monophosphate (cAMP)

Cells were lysed with moderate shaking in 0.1 M HCl for 15 min at room temperature before cellular debris was removed by centrifugation at 600 g. The cAMP content in cellular lysates was determined by a competitive enzyme immunoassay as directed by the manufacturer's instructions (Cyclic AMP (Direct) Enzyme Immunometric Assay (EIA), non-acetylated format, Assay Designs®, Ann Arbor, MI). The absorption of each sample was quantified at 405 nm by a SpectraMax® Plus384 plate reader (Molecular Devices). Each value was normalized to internal controls and a freshly prepared standard curve ranging from 0.2 - 200 pmol cAMP/ml.

Cytotoxicity assay

Cytotoxicity was determined by the colorimetric Cytotoxicity Detection Kit (Roche Applied Science, Penzberg, Germany), which measures the activity of lactate dehydrogenase (LDH), released from the cytosol of damaged cells. In brief, cell culture supernatants were taken at

different time points after induction of hypoxia, added to LDH substrate, and incubated for 30 min at 25°C. The absorbance at 490 nm was measured by a SpectraMax[®] Plus384 spectrophotometer with a reference wavelength of 690 nm. LDH values corresponding to 100% neuronal death were established by addition of 1% Triton-X 100 to untreated control cells, which induced total cell lysis and maximal LDH release. Determined LDH values were displayed as a percentage of neuronal death compared to total cell lysis.

Caspase-activity assay

SK-N-SH cells were lysed in 10 mM HEPES-KOH/pH 7.9, 350 mM NaCl, 1% Nonidet P-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM dithiothreitol, 2.5 mM phenylmethylsulfonyl fluoride, and 20 µg/ml aprotinin. Extracts were diluted 1:10 in 100 mM HEPES-KOH/pH 7.5, containing 2 mM dithiothreitol and 60 µM of the fluorogenic caspase-3 substrate N-acetyl-DEVD-7-amin-4-methylcoumarin (Ac-DEVD-AMC; Enzo Life Sciences, Loerrach, Germany). Caspase-3 like activity was determined by a Gemini XS plate reader at 340/460 nm (Molecular Devices) for 30 min at 27°C. Values were normalized to protein content and expressed as increasing relative light units (RLU) per second.

Measurement of intracellular ATP

Relative intracellular ATP-content was determined by the bioluminescence based ATP Assay Kit (Calbiochem, Merck KGaA, Darmstadt, Germany), that quantifies the ATP-dependent oxidation of luciferin by luciferase. Briefly, cells were lysed in 250 µl ice-cold ATP-releasing buffer containing 20 mM Tris-HCl/pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Nonidet P-40, 100 µg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 70 µg/ml of aprotinin by repeated freeze/thaw cycles. The cell lysates were diluted 1:5 by addition of 40 µl nucleotide releasing buffer and luciferase activity was determined for 10 seconds per sample upon automated injection of 50 µl of a

luciferin/luciferase mixture using a luminometer (MicroLuminat Plus LB 96P; Berthold Technologies, Bad Wildbad, Germany). Values were expressed as relative light units (RLU) and normalized to protein content, that was determined by the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Thermo Fisher Scientific, Rockford, IL).

Statistical Analysis

Results are expressed as means \pm standard deviations for the indicated number of separate experiments. Statistical differences between experimental groups were determined by performing one-way ANOVA followed by the Dunnett's multiple comparisons test, unpaired t test, or by two-way ANOVA, followed by the Bonferroni's *post hoc* test as indicated. Differences between groups were considered to be significant at $P < 0.05$. Statistical analyses were carried out using the Prism software package (GraphPad Software Inc., La Jolla, CA).

Results

Carbimazole inhibits global protein synthesis by induction of eEF2 phosphorylation

Translational elongation of the polypeptide chain is regulated by eEF2, a translocase that is responsible for the movement of the mRNA along the ribosome (Jorgensen *et al.*, 2006). The activity of eEF2 is tightly controlled by posttranslational modification (Carlberg *et al.*, 1990) and phosphorylation of eEF2 at Thr56 is associated with translational repression (Ryazanov *et al.*, 1988). To determine whether carbimazole affects translational elongation, human neuronal SK-N-SH cells were treated with increasing doses of carbimazole and eEF2 phosphorylation was analyzed by immunoblotting. Our results clearly indicate that phosphorylation of eEF2 at Thr56 is induced by carbimazole in a dose dependent manner (Fig. 1A, Supplemental Fig. 1A). The inhibitory modification of eEF2 was already detectable at 50 μ M and was markedly pronounced in the presence of ≥ 0.5 mM carbimazole. A time kinetic demonstrated, that phosphorylation of eEF2 was induced within 10 – 60 min and persisted for at least 24 h (Fig. 1B, Supplemental Fig. 1B). Under these conditions carbimazole mediated no cytotoxic effects, because a significant increase in intracellular LDH activity was not detected in cell culture supernatants of SK-N-SH cells that were cultured in the presence or absence of carbimazole for 72 h (Fig. 3D/5B). The total amount of eEF2 was unchanged by exposure to carbimazole, indicating that increases in phospho-eEF2 are due to enzymatic modification but not increased protein synthesis (Fig. 1A/B, lower blots).

Posttranslational modification of translational regulators is a common mechanism to control *de novo* protein synthesis (Browne and Proud, 2002). To determine, whether carbimazole-dependent phosphorylation of eEF2 at Thr56 corresponds to translational inhibition, SK-N-SH cells were metabolically labeled with [³⁵S]methionine for 2 h, and the amount of *de novo* synthesized polypeptides was visualized by SDS-PAGE and autoradiography. Preliminary experiments ensured that incorporation of [³⁵S]methionine into the growing polypeptide chains was directly proportional to the length of labeling time within 20 min to 6 h (data not

shown). As shown in Fig. 2A/B, exposure of SK-N-SH cells to carbimazole resulted in a dose dependent reduction of the basal rate of protein synthesis. Treatment of cells for 4 h with cycloheximide, a known inhibitor of translation, also resulted in a marked decrease in protein synthesis (Fig. 2A/B). In contrast, total protein levels visualized by Coomassie G-250 staining of gels before autoradiography were similar, demonstrating the comparison of equivalent samples and excluding cytotoxic effects of the tested compounds (Fig. 2C). These findings indicate that carbimazole does not selectively downregulate protein expression but instead exerts a global inhibitory effect on protein translation. The ability of carbimazole to decrease global protein synthesis is consistent with the observed increase in eEF2 phosphorylation.

Carbimazole moderately elevates cytoplasmic calcium levels

Next, the mechanism involved in translational regulation by carbimazole was investigated. eEF2 phosphorylation at Thr56 is mediated by eEF2K (Nairn and Palfrey, 1987). To prove that eEF2K is directly involved in carbimazole mediated phosphorylation of eEF2, SK-N-SH cells were incubated with NH125, a specific eEF2K inhibitor. As shown in Fig. 3A (densitometry in Supplemental Fig. 2A), NH125 abrogated eEF2 phosphorylation in the presence of carbimazole, confirming that this highly specific kinase is crucial for carbimazole mediated translational regulation.

Because eEF2K is dependent on calcium and calmodulin for activity (Ryazanov, 1987), we next analyzed the effect of carbimazole on cytoplasmic calcium content. Free intracellular calcium imaging was performed by labeling SK-N-SH cells with the membrane permeable fluorometric calcium indicator fura-2AM. As illustrated in Fig. 3B, carbimazole increased the fura-2 fluorescence by 4 to 6-fold. This increase was dependent on the presence of extracellular calcium, indicating the opening of plasma membrane gated calcium channels. In contrast, thapsigargin elevated cytoplasmic calcium levels also in the absence of extracellular calcium, thus demonstrating the depletion of intracellular calcium stores.

Intracellular calcium overload is a critical event in excitotoxicity (Won *et al.*, 2002). Therefore, caspase-3 activity assays were performed to analyze whether elevated calcium levels due to carbimazole or thapsigargin treatment induce apoptosis. As shown in Fig. 3C, thapsigargin induced strong caspase-3 like activity, whereas carbimazole mediated no significant cleavage of the caspase-3 specific substrate Ac-DEVD-AMC. Similarly, lactate dehydrogenase leakage into cell culture supernatants, used as an unspecific marker for cell injury, was only observed in the presence of thapsigargin (Fig. 3D). This indicates that the moderate increase in intracellular calcium by carbimazole treatment does not facilitate cytotoxicity but may be more likely involved in calcium-dependent intracellular signaling.

Protein kinases in the regulation of eEF2 phosphorylation

Calcium in resting cells is about 0.1 μM and the observed 4 to 6 fold increase might be close to optimum, described for the induction of calcium/calmodulin-dependent proteins (Diggle *et al.*, 1998). To further characterize the biological consequences of a carbimazole-dependent intracellular calcium increase, the calcium/calmodulin antagonists CGS 9343B or camstatin were used. Surprisingly, neither CGS 9343B nor camstatin influenced carbimazole-dependent eEF2 phosphorylation, arguing for a calcium/calmodulin-independent eEF2 modulation by carbimazole (Fig. 4A, Supplemental Fig. 2B/C).

Several protein kinases regulate eEF2K activity (Browne and Proud, 2002). Phosphorylation of eEF2K by cAMP-dependent protein kinase (PKA) results in acquisition of calcium independent eEF2K activity and phosphorylation of eEF2 (Redpath and Proud, 1993). In fact, accumulation and release of cAMP as a response to thyroid-stimulating hormone and thyroid-stimulating antibodies have been described, but direct effects of carbimazole on adenylate cyclase and cAMP levels remained unidentified (Bidey *et al.*, 1981). Therefore we examined whether carbimazole affects cAMP signaling and determined the amount of cAMP in carbimazole treated SK-N-SH cells by a competitive enzyme immunoassay. We observed a

transient increase in cytosolic cAMP in neuronal SK-N-SH cells 60 min after carbimazole treatment (Fig. 4B). However, the biological significance of this marginal increase is unclear. For comparison, 100 μ M of the cAMP analog 8-(4-chlorophenylthio)-cAMP is used to induce biological responses (Hovland *et al.*, 1999) and when we used forskolin, a labdane diterpen that is commonly applied to induce adenylate cyclase dependent cAMP synthesis, cAMP levels were increased 100-200 times higher than with carbimazole (data not shown). In order to determine the relevance of the observed carbimazole-mediated cAMP increase, we applied the cell permeable cAMP analog cAMPS-Rp or the myristoylated peptide inhibitor PKI 14-22 amide as specific inhibitors of PKA and subsequently measured PKA-independent eEF2 phosphorylation in the presence of carbimazole. As demonstrated in Fig. 4C (densitometry in Supplemental Fig. 2D/E), treatment of SK-N-SH cells with cAMPS-Rp or PKI 14-22 amide had no effect on carbimazole-dependent eEF2 phosphorylation, indicating that the cAMP/PKA pathway is not directly involved in translational regulation by carbimazole.

In contrast to PKA, the mTOR pathway induces eEF2-dependent translational elongation by inhibitory phosphorylation of eEF2K (Browne and Proud, 2002). To determine, whether inactivation of eEF2K by mTOR is reversed by carbimazole, carbimazole-mediated eEF2 phosphorylation was analyzed in the presence of the specific mTOR inhibitor rapamycin. As shown in Fig. 4D (Supplemental Fig. 2F), treatment of cells with rapamycin had no effect on carbimazole-dependent eEF2 phosphorylation, indicating that the mTOR pathway is also not participating in translational regulation by carbimazole.

Carbimazole induces AMP-dependent protein kinase (AMPK)

Various reports indicate that heterocyclic thiourylenes attenuate mitochondrial respiration (Aldridge and Parker, 1960; Chance *et al.*, 1963). Energy starvation may then induce AMPK, an enzyme that is critically involved in the regulation of energy homeostasis (Oakhill *et al.*, 2012). Since carbimazole is a heterocyclic thiourylene derivate, we speculated that an

interaction of this compound with oxidative phosphorylation might lead to a shift in cellular AMP/ATP-ratios and consequently in AMPK/eEF2K-dependent phosphorylation of eEF2. To test this hypothesis, AMPK-activity was determined in the presence of carbimazole by visualizing AMPK autophosphorylation at Thr172 in the activation loop, which is required for activity (Oakhill *et al.*, 2012). As shown in Fig. 4E (densitometry in Supplemental Fig. 1C), exposure of neuronal SK-N-SH cells to carbimazole revealed a marked increase in the levels of Thr172-phosphorylated AMPK 1 h after onset of treatment, which persisted for at least 6 h. The total amount of AMPK was unchanged by exposure to carbimazole, indicating that increases in phospho-AMPK are due to enzymatic modification and not enhanced protein synthesis (Fig. 4E, lower blot).

To investigate the significance of AMPK in eEF2 inhibition, we compared the phosphorylation pattern of eEF2 at Thr56 in the presence or absence of dorsomorphin dihydrochloride, a specific AMPK inhibitor. As shown in Fig. 4F (densitometry in Supplemental Fig. 2G), dorsomorphin hydrochloride completely blocked carbimazole-induced eEF2 phosphorylation, confirming AMPK/eEF2K-dependent eEF2 inactivation.

Carbimazole protects neuronal SK-N-SH cells from hypoxic cell damage

Secondary brain damage, following hypoxic cell injury evolves progressively and thus offers a therapeutic window for pharmacologic intervention (Heiss and Graf, 1994). To analyze, whether carbimazole protects neurons from hypoxia-related cell damage, SK-N-SH cells were exposed to oxygen-deprivation before intracellular lactate dehydrogenase release into cell culture medium was measured as an index of neuronal injury. As shown in Fig. 5A, 48 h of hypoxia induced significant cell injury as indicated by marked LDH leakage. Surprisingly, we observed that cell damage due to oxygen-deprivation was most pronounced in serum activated neuronal SK-N-SH cells, whereas serum starved cells remained largely unaffected. The difference in susceptibility implies that metabolically active cells are particularly vulnerable

to hypoxic cell death in contrast to resting cells, which may be resistant to injury by oxygen-deprivation. Therefore we suspected that the observed translational arrest by carbimazole may contribute to metabolic repression and thus affects hypoxic cell damage in otherwise non-restricted cells. To confirm our assumption, cell damage was assessed in carbimazole treated, oxygen-deprived SK-N-SH cells. As demonstrated in Fig. 5B, hypoxia-induced cell injury was almost completely abolished by carbimazole 48 h after onset of deoxygenation. At this time point, carbimazole reduced the extent of hypoxia-mediated LDH leakage to levels comparable with the spontaneous release of untreated, normoxic control cells. However, carbimazole-mediated protection decreased after 72 h, probably due to a limited half life of carbimazole in serum conditioned medium. The morphological studies confirmed the results of LDH leakage, i.e., hypoxia induced detachment of SK-N-SH cells and thus decreased viable cellular density, whereas carbimazole prevented detachment of neurons and cellular density was comparable to untreated normoxic control cells (data not shown).

Inhibition of global protein synthesis by carbimazole preserves availability of ATP

ATP-depletion has deleterious effects on ion homeostasis and energy metabolism, resulting in glycolysis-dependent acidosis, excitotoxicity, and neuronal cell death (Won *et al.*, 2002). Protein synthesis is a dominant energy consuming cellular process and suppression of translation might result in substantial bioenergetic savings (Hand and Hardewig, 1996). Therefore we investigated whether carbimazole-mediated translational repression affected intracellular ATP-content in normoxic or oxygen-deprived SK-N-SH cells, using an ATP-dependent bioluminescence assay (Fig. 6). Carbimazole did not significantly reduce availability of ATP in normoxic cells as demonstrated by comparable luciferase activity in cell lysates of non-treated versus carbimazole-treated SK-N-SH cells. This is surprising, as AMPK, a most sensitive detector for increased AMP/ATP ratios, was activated in the presence of carbimazole (Fig. 4E, Supplemental Fig. 1C). However, in oxygen-deprived cells,

maximal ATP-depletion was already detectable after 12 h, with no further significant reduction for the next 60 h in hypoxic cell culture medium. Carbimazole prevented hypoxia-dependent ATP-deprivation in an early phase for up to 48 h. The ATP-dependent bioluminescence was only insignificantly reduced in lysates of carbimazole treated, hypoxic SK-N-SH cells, when compared to normoxic control conditions. This indicates that translational repression by carbimazole is linked to the conservation of high-energy phosphates in form of ATP, thus opposing hypoxic cell injury. At 72 h, ATP-availability in carbimazole-treated hypoxic cells was still considerably increased when compared to oxygen-deprived control cells but noticeably reduced in comparison to normoxic cells. The observed reduction in ATP-availability in carbimazole-treated hypoxic cells may be due to a time dependent loss of carbimazole activity and correlates with the reduced protection from hypoxic damage (Fig. 5B). In conclusion, conservation of cellular energy by inhibition of global protein synthesis and its reallocation to essential ATP-consuming processes may be the key for cell survival under conditions of decreased oxygen levels as ATP-supply is limited.

Hypoxia induces late and transient phosphorylation of eEF2 and AMPK

Stroke itself induces marked reductions in protein synthesis, which might limit the therapeutic benefit of an additional protein synthesis inhibitor. Therefore we tested whether hypoxia resulted in eEF2 and AMPK phosphorylation. Our observations depicted in Fig. 7 show, that oxygen deprivation induced both phosphorylation of eEF2 and AMPK (densitometry in Supplemental Fig. 3A/B). However these posttranslational modifications were a late and transient event. At that time hypoxia-induced ATP-depletion was already maximal (Fig. 6), which indicates that translational arrest by hypoxia is induced as a consequence of energy deprivation and therefore cells are unable to maintain energy homeostasis. Indeed, at the time of hypoxia-induced eEF2 and AMPK phosphorylation neurotoxicity due to oxygen deprivation was highly pronounced and could not be further prevented (Fig.5). These results

indicate that adaptive preservation of energy is necessary before ATP-depletion by hypoxia is complete.

Translational inhibitors inhibit hypoxic cell injury and preserve intracellular ATP-content

Since there is evidence that carbimazole may inhibit neuronal apoptosis independently of translational repression (Humar *et al.*, 2009), we asked whether other translational inhibitors similarly reduce hypoxic cell damage. As shown in Fig. 8A, the protein synthesis inhibitor cycloheximide completely abrogated hypoxic injury for 72 h. In the presence of cycloheximide the amount of lactate dehydrogenase was comparable in supernatants of normoxic and oxygen-deprived cells. Co-incubation with anisomycin also prevented neuronal hypoxic damage but displayed biphasic protection (Fig. 8B). In an early phase of hypoxia (\leq 48 h) anisomycin (1 μ g/ml) was most efficient in preventing hypoxic damage, whereas after 72 h lower doses of anisomycin (10 ng/ml) were necessary to maintain protection, probably due to a toxic effect of persistent anisomycin treatment. In fact, high dose anisomycin treatment has been associated with exacerbated neuronal injury (Hong *et al.*, 2007). Accordingly, we observed that LDH-leakage in the presence of high anisomycin doses was comparable in normoxic and hypoxic SK-N-SH cells (Fig. 8B). In conclusion, these results demonstrate the therapeutic potential of translational inhibitors in neuroprotection during hypoxia but indicate that their therapeutic dose must be carefully titrated according to the temporal progression of the injury process.

Next we investigated, whether neuroprotection by cycloheximide or anisonmycin also correlated with a stabilization of ATP-content in hypoxic SK-N-SH cells. As shown in Fig. 8C/D, we observed that hypoxic neuronal SK-N-SH cells displayed a massive loss in availability of intracellular ATP, which corresponds to the experiments shown in Fig. 6. However, cycloheximide completely prevented ATP-depletion following oxygen deprivation. In fact, ATP-levels in cycloheximide treated cells were considerably increased when

compared to untreated normoxic control extracts. Anisomycin also significantly ameliorated ATP-depletion following oxygen deprivation. However, maintenance of cellular energy supply by anisomycin was time and concentration dependent and correlated with the biphasic protection against hypoxic injury, as observed in Fig. 8B. High dose anisomycin treatment was especially effective in the beginning of hypoxic injury; however, a long lasting ATP-supply was only sustained when low therapeutical doses of anisomycin were used, indicating cytotoxic side effects of this compound.

The preservation of intracellular ATP-levels in cycloheximide or anisomycin treated hypoxic cells generally correlated with protection from hypoxic cell damage. Based on these observations we postulate that conservation of cellular energy by inhibition of global protein synthesis and its reallocation to essential ATP-consuming processes provides a promising therapeutic approach to rescue potentially vulnerable hypoxic cells when ATP-generation is limited.

Translational control by carbimazole in primary cortical neurons

The rate of translation in primary cortical neurons and SK-N-SH cells might significantly vary. Furthermore, considering that neurons are highly differentiated cells, with translation occurring both in the soma and dendrites, protein inhibition in these two compartments may have differential effects in cell death or cell survival that will probably not become visible in SK-N-SH cells. Therefore, primary cortical cell cultures were established to analyze the effects of carbimazole on regulatory posttranslational modifications of eEF2 and AMPK in terminally differentiated neurons. As observed in Fig. 9A, carbimazole induced a robust phosphorylation of eEF2 (densitometry in Supplemental Fig. 3C). Phosphorylation of AMPK followed a similar kinetic, indicating that the posttranslational modification of eEF2 and AMPK are both regulated by a common pathway (Supplemental Fig. 3C/D).

Because eEF2 phosphorylation is associated with translational repression, we next analyzed whether carbimazole also inhibits protein synthesis in primary differentiated neurons. Metabolic labeling of cortical neurons with [³⁵S]methionine indicated that global protein synthesis is inhibited by carbimazole (Fig. 9B, Supplemental Fig. 4), which correlated with eEF2 and AMPK phosphorylation (Fig. 9A, Supplemental Fig. 3C/D).

In SK-N-SH cells, the inactivating modification of eEF2 and translational repression preserved intracellular energy content and ameliorated hypoxic damage (Fig. 5/6). To test whether pharmacologic inhibition of protein synthesis also reduces hypoxic injury of differentiated primary neurons, cortical cell cultures were exposed to hypoxia in the presence or absence of carbimazole and cell damage was assessed by an LDH-release assay. In analogy to SK-N-SH cells oxygen deprivation resulted in significant neurotoxicity (Fig. 9C). Cell death was independent of caspase-3, whereas pharmacologic induction of calcium overload by thapsigargin induced pro-caspase 3 cleavage, PARP cleavage, and eEF2 phosphorylation (Fig. 9D, Supplementary Fig. 3E-G). This indicates that loss of ion homeostasis, as observed during excitotoxicity, might also induce translational arrest. However, translational arrest induced by hypoxia or calcium overload will be inefficient to salvage neurons because at this time cell injury is already extensive (Fig. 9C) and the progression of programmed cell death following caspase activation is thought to be irreversible.

In analogy to SK-N-SH cells, damage of cortical neurons by oxygen withdrawal was significantly ameliorated by carbimazole (Fig. 9C). To test whether cytoprotection by carbimazole is mediated by the maintenance of energy balance in hypoxic cortical neurons, cell lysates were analyzed for their ATP content (Fig. 9E). Hypoxia resulted in a significant decline of ATP levels in cortical neurons but the depletion of ATP in the absence of oxygen was significantly diminished by carbimazole. In conclusion these results confirm that conservation of cellular energy by inhibition of global protein synthesis by carbimazole is

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neuroprotective in both SK-N-SH cells and terminally differentiated cortical neurons, especially when oxygen supply is limited.

Discussion

In our study we have shown that carbimazole diminishes hypoxic neuronal damage by inhibiting global protein synthesis. Cycloheximide and anisomycin also reduced cellular damage of oxygen-deprived neurons, indicating that repressors of translation are potential candidates for treatment of cerebral hypoxia and ischemia.

Protein synthesis is a major consumer of cellular energy (Rolfe and Brown, 1997). Under conditions of limited oxygen availability repression of translation may result in energy conservation, which can instead be used to maintain cellular mechanisms necessary for survival. We observed that translational repressors prevented or significantly delayed the depletion of ATP-stores in oxygen-deprived neurons. In addition, independent studies demonstrate that molecules which maintain mitochondrial respiratory activity during hypoxia or extrinsic application of high-energy metabolites also ameliorate ischemic brain damage (Bouaziz *et al.*, 2002; Ying *et al.*, 2007). Our observations may also explain neuroprotective effects mediated by therapeutic hypothermia. Therapeutic hypothermia has been associated with the attenuation of deleterious cascades, many of them dependent on ATP-availability or translation, including the regulation of cerebral metabolism, excitotoxicity, inflammation, the integrity of the blood-brain barrier, and acidosis (Moore *et al.*, 2011).

Translational inhibition by cycloheximide and anisomycin are well characterized, whereas the clinical use of carbimazole is primarily based upon thyroid peroxidase and dehalogenase inhibition. Here we demonstrate that carbimazole additionally interferes with translational elongation by inducing a specific modification of eEF2. Phosphorylation of eEF2 at Thr56 prevents translocation of the nascent protein chain from the A-site to the P-site of the ribosome (Carlberg *et al.*, 1990; Ryazanov *et al.*, 1988) and offers a plausible explanation for the observed translational repression and conservation of intracellular energy. Furthermore we could establish the molecular mechanism responsible for carbimazole-mediated eEF2 phosphorylation, which includes AMPK-dependent activation of eEF2K. Interestingly, both

kinases are associated with cytoprotective molecular mechanisms, including reduced hypoxic injury, stress resistance, autophagy, inhibition of plaque formation in Alzheimer's disease, regulation of energy homeostasis, and endothelial NO synthesis that improves blood flow in ischemic brain tissue (Greco *et al.*, 2009;Hardie, 2004;Li and McCullough, 2010;Py *et al.*, 2009;Terai *et al.*, 2005;Zhang *et al.*, 2010). However, whether carbimazole exerts all of these protective mechanisms remains to be established.

Carbimazole-mediated effects on translation were observed beginning at concentrations 5 times higher than serum levels of thionamides typically found during treatment of hyperthyroidism (Skellern *et al.*, 1974). However, patients obtain long term anti-thyroid treatment which may last for years and the turnover of thionamides is slow in tissue compared to a short plasma half-life (Jansson *et al.*, 1983). This might result in drug accumulation as demonstrated for related agents (Turcant *et al.*, 1985).

Structural analogues of carbimazole inhibit respiration and uncouple oxidative phosphorylation (Aldridge and Parker, 1960). We did not directly analyze the effects of carbimazole on cellular respiration, however, we observed activation of AMPK, an enzyme that monitors intracellular AMP/ATP ratios, which are naturally increased when generation of energy is impaired. However, ATP-levels were not noticeably reduced by carbimazole, which corresponds to other studies, demonstrating that inhibition of respiration by thiourylenes is incomplete and does not appreciably depress ATP-levels under basal metabolic conditions (Aldridge and Parker, 1960;Chance *et al.*, 1963).

Anisomycin also efficiently reduced hypoxic cell damage, but whether protection is based on translational arrest or modulation of p38 MAP kinase activity (Hong *et al.*, 2007) is unclear. We observed that the p38 inhibitor SB202190 reduced neuronal cell damage during oxygen-deprivation or calcium-overload (Matjaz Humar, unpublished observation) and we previously described that carbimazole inhibits p38 activation (Humar *et al.*, 2007). However, protection by SB202190 was less pronounced and restricted to a short time frame, indicating that in

addition to inhibition of p38, anisomycin or carbimazole must activate further protective mechanisms, e.g. translational repression and reduction of cellular energy demand.

Stroke itself induces marked reductions in protein synthesis, which could limit the therapeutic benefit of an additional protein synthesis inhibitor. However, our data indicates that translational repression by hypoxia occurs as a late and transient event. Consequently, eEF2 phosphorylation due to hypoxia is inefficient as a protective mechanism, because ATP pools are already irreversibly depleted and the residual intracellular energy supply is not sufficient to salvage oxygen-deprived neurons. Furthermore, we observed that intracellular calcium overload, typical for excitotoxicity, favored eEF2 phosphorylation and apoptosis, confirming that translational arrest during stroke is induced when neuronal damage is already irreversible. However, pharmacologic preconditioning of neurons by translational inhibitors reduces cellular metabolism and ATP-consumption before ATP-pools are critically depleted and before neurons are severely damaged due to hypoxia. This may limit progressive tissue damage in the penumbra that evolves over hours or even days after stroke. Moreover it was shown that carbimazole inhibits calcium-dependent caspase-3 activation (Humar et al., 2009). Our study demonstrated the pharmacologic prevention of hypoxic damage, which involves the induction of AMPK. However, AMPK is also critical for nutrient sensing and during stroke nutrient restriction contributes to increased AMP/ATP ratios and neuronal tissue damage. Recently it has been described that nutrient restriction may result in translational repression. In addition, this pathway is exploited by tumor cells in adapting to metabolic stress (Leprivier et al., 2013). Induction of AMPK by carbimazole may provide a survival benefit before it is too late to restore energy balance and thus may prevent the progressive expansion of penumbral tissue damage during stroke in close analogy to hypoxia-induced injury.

Hypoxic brain damage depends on several independent pathways. Selective pharmacological inhibition of a single pathway will most likely be insufficient in preventing the deleterious effects of hypoxic brain damage (Stankowski and Gupta, 2011; White *et al.*, 2000). Clinical

trials with brain-injured patients also showed poor clinical efficacy when pharmacologic intervention was applied at a single target (Labiche and Grotta, 2004). Carbimazole and other structurally related compounds exert a wide array of cytoprotective effects. They ameliorate the consequences of calcium overload by preventing the activation of calcium dependent enzymes and PP2B-dependent neuronal apoptosis (Humar *et al.*, 2009). Furthermore they inhibit inflammation and induce a cytoprotective heat shock response (Humar *et al.*, 2008;Roesslein *et al.*, 2008). Drugs that downregulate many independent deleterious pathways should be more efficient in limiting secondary neuronal tissue damage than drugs that inhibit only single targets.

Bioenergetic conservation might not be the only cytoprotective mechanism attributed to translational arrest. Hypoxic cell injury is also determined by translation of inflammatory mediators, inducible nitric oxide synthase, cyclooxygenase-2, or matrix metalloproteinases (Cunningham *et al.*, 2005;White *et al.*, 2000;Won *et al.*, 2002). Inhibition of global protein synthesis could prevent their translation, further reducing neuronal damage.

The pathophysiology of several diseases is based on translation and overexpression of damage-inducing proteins, such as in hypertrophy, inflammation, malignant transformation, or structural remodelling. Inhibition of protein synthesis may present an intriguing therapeutic option to alleviate characteristic symptoms of these diseases. Previously, we have demonstrated that translational inhibition results in the inhibition of cell cycle progression (Schwer *et al.*, 2013), which may slow down hyperproliferative diseases like cancer and carbimazole treatment has been associated with deceleration of tumour progression in some patients (Jurkovic K., Oncological diseases: Treatment by Blocking Tumour Metabolism Treatment of Mastopathia Fibrosa. <http://www.docstoc.com/docs/73386197>). On the other hand, general inhibition of protein synthesis might prevent cellular regeneration and tissue repair. Interestingly, many cytoprotective proteins are preferentially synthesized during stress conditions associated with translational arrest (Hernandez *et al.*, 2004;Yueh and Schneider,

2000). We observed that some carbimazole-analogues induce HSP70, which was associated with cytoprotection (Roesslein *et al.*, 2008). Because tissue repair is a late event following organ-damage, we suggest first to minimize tissue damage by active pharmaceutical intervention, because the therapeutic restoration of neuronal tissue is unsolved. However, at a later time point, translation needs to be restored to allow tissue repair based on the synthesis of vital proteins. Further studies will be necessary to establish a balanced therapeutic approach to this dynamic process.

Thiopental, a structural analogue of carbimazole, also promotes neuroprotection (Nordby and Nesbakken, 1984). Thiopental induces phosphorylation of eEF2, translational repression and protection from hypoxic cell death, indicating that modulation of this cellular pathway is a characteristic feature of heterocyclic thiourylenes. Oxy-derivates of thiourylenes display a significantly reduced ability to perform translational repression, demonstrating that the modulation of intracellular signal transduction significantly depends on the sulphur side chain of the thiourea group (Matjaz Humar, unpublished observations). Derivates of heterocyclic thiourylenes may reveal a pharmacologically relevant scaffold for the development of novel organ-protective compounds.

In summary, the presented findings suggest that carbimazole and other inhibitors of translation protect neurons from hypoxic cell-injury by preserving the ATP-content during conditions of limited oxygen availability. Carbimazole-mediated translational repression and energy conservation was based on the activation of AMPK, resulting in eEF2 phosphorylation and inactivation. Thus, heterocyclic thiourylenes may represent interesting candidates for the development of new organ-protective compounds. Furthermore, our results suggest the evaluation of other general translational inhibitors, agents that maintain mitochondrial respiratory activity during hypoxia, and the therapeutic application of high-energy molecules as potential strategies to ameliorate hypoxic tissue damage.

Authorship Contributions

Participated in research design: Humar

Conducted experiments: Lehane, Guelzow, Zenker, and Humar

Performed data analysis: Lehane, Guelzow, Schwer, Erxleben, and Humar

Wrote or contributed to the writing of the manuscript: Lehane, Schwer, Heimrich, Buerkle,
and Humar

References

- Aldridge WN and Parker VH (1960) Barbiturates and Oxidative Phosphorylation. *Biochemical Journal* **76**:47-56.
- Barreto AD (2011) Intravenous thrombolytics for ischemic stroke. *Neurotherapeutics* **8**:388-399.
- Bidey SP, Marshall NJ, and Ekins RP (1981) Adenylate cyclase activity and the accumulation and release of adenosine 3',5'-monophosphate in normal human thyroid tissue slice preparations: responses to thyrotropin and thyroid-stimulating antibodies. *J Clin Endocrinol Metab* **53**:246-253.
- Bouaziz N, Redon M, Quere L, Remacle J, and Michiels C (2002) Mitochondrial respiratory chain as a new target for anti-ischemic molecules. *Eur J Pharmacol* **441**:35-45.
- Boutilier RG and St Pierre J (2000) Surviving hypoxia without really dying. *Comp Biochem Physiol A Mol Integr Physiol* **126**:481-490.
- Browne GJ and Proud CG (2002) Regulation of peptide-chain elongation in mammalian cells. *Eur J Biochem* **269**:5360-5368.
- Carlberg U, Nilsson A, and Nygard O (1990) Functional properties of phosphorylated elongation factor 2. *Eur J Biochem* **191**:639-645.
- Chan AY, Soltys CL, Young ME, Proud CG, and Dyck JR (2004) Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. *J Biol Chem* **279**:32771-32779.

- Chance B, Williams GR, and Hollunger G (1963) Inhibition of electron and energy transfer in mitochondria. I. Effects of Amytal, thiopental, rotenone, progesterone, and methylene glycol. *J Biol Chem* **238**:418-431.
- Cunningham LA, Wetzel M, and Rosenberg GA (2005) Multiple roles for MMPs and TIMPs in cerebral ischemia. *Glia* **50**:329-339.
- Diggle TA, Redpath NT, Heesom KJ, and Denton RM (1998) Regulation of protein-synthesis elongation-factor-2 kinase by cAMP in adipocytes. *Biochemical Journal* **336**:525-529.
- Du J, Guan T, Zhang H, Xia Y, Liu F, and Zhang Y (2008) Inhibitory crosstalk between ERK and AMPK in the growth and proliferation of cardiac fibroblasts. *Biochem Biophys Res Commun* **368**:402-407.
- Furlan M, Marchal G, Viader F, Derlon JM, and Baron JC (1996) Spontaneous neurological recovery after stroke and the fate of the ischemic penumbra. *Ann Neurol* **40**:216-226.
- Greco SJ, Sarkar S, Johnston JM, and Tezapsidis N (2009) Leptin regulates tau phosphorylation and amyloid through AMPK in neuronal cells. *Biochem Biophys Res Commun* **380**:98-104.
- Hand SC and Hardewig I (1996) Downregulation of cellular metabolism during environmental stress: mechanisms and implications. *Annu Rev Physiol* **58**:539-563.
- Hardie DG (2004) The AMP-activated protein kinase pathway--new players upstream and downstream. *J Cell Sci* **117**:5479-5487.
- Heiss WD and Graf R (1994) The ischemic penumbra. *Curr Opin Neurol* **7**:11-19.

- Hernandez G, Vazquez-Pianzola P, Sierra JM, and Rivera-Pomar R (2004) Internal ribosome entry site drives cap-independent translation of reaper and heat shock protein 70 mRNAs in *Drosophila* embryos. *RNA* **10**:1783-1797.
- Hong SS, Qian H, Zhao P, Bazy-Asaad A, and Xia Y (2007) Anisomycin protects cortical neurons from prolonged hypoxia with differential regulation of p38 and ERK. *Brain Res* **1149**:76-86.
- Horman S, Browne G, Krause U, Patel J, Vertommen D, Bertrand L, Lavoinnie A, Hue L, Proud C, and Rider M (2002) Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. *Curr Biol* **12**:1419-1423.
- Hovland R, Eikhom TS, Proud CG, Cressey LI, Lanotte M, Doskeland SO, and Houge G (1999) cAMP inhibits translation by inducing Ca²⁺/calmodulin-independent elongation factor 2 kinase activity in IPC-81 cells. *FEBS Lett* **444**:97-101.
- Humar M, Dohrmann H, Stein P, Andriopoulos N, Goebel U, Heimrich B, Roesslein M, Schmidt R, Schwer CI, Hoetzel A, Loop T, Pahl HL, Geiger KK, and Pannen BH (2007) Repression of T-cell function by thionamides is mediated by inhibition of the activator protein-1/nuclear factor of activated T-cells pathway and is associated with a common structure. *Mol Pharmacol* **72**:1647-1656.
- Humar M, Dohrmann H, Stein P, Andriopoulos N, Goebel U, Roesslein M, Schmidt R, Schwer CI, Loop T, Geiger KK, Pahl HL, and Pannen BH (2008) Thionamides inhibit the transcription factor nuclear factor-kappaB by suppression of Rac1 and inhibitor of kappaB kinase alpha. *J Pharmacol Exp Ther* **324**:1037-1044.

- Humar M, Graetz C, Roesslein M, Goebel U, Geiger KK, Heimrich B, and Pannen BH (2009) Heterocyclic thioureylenes protect from calcium-dependent neuronal cell death. *Mol Pharmacol* **75**:667-676.
- Jansson R, Dahlberg PA, Johansson H, and Lindstrom B (1983) Intrathyroidal concentrations of methimazole in patients with Graves' disease. *J Clin Endocrinol Metab* **57**:129-132.
- Jorgensen R, Merrill AR, and Andersen GR (2006) The life and death of translation elongation factor 2. *Biochem Soc Trans* **34**:1-6.
- Labiche LA and Grotta JC (2004) Clinical trials for cytoprotection in stroke. *NeuroRx* **1**:46-70.
- Leprivier G, Remke M, Rotblat B, Dubuc A, Mateo AR, Kool M, Agnihotri S, El Naggar A, Yu B, Somasekharan SP, Faubert B, Bridon G, Tognon CE, Mathers J, Thomas R, Li A, Barokas A, Kwok B, Bowden M, Smith S, Wu X, Korshunov A, Hielscher T, Northcott PA, Galpin JD, Ahern CA, Wang Y, McCabe MG, Collins VP, Jones RG, Pollak M, Delattre O, Gleave ME, Jan E, Pfister SM, Proud CG, Derry WB, Taylor MD, and Sorensen PH (2013) The eEF2 kinase confers resistance to nutrient deprivation by blocking translation elongation. *Cell* **153**:1064-1079.
- Li J and McCullough LD (2010) Effects of AMP-activated protein kinase in cerebral ischemia. *J Cereb Blood Flow Metab* **30**:480-492.
- Lin CC, Wu YJ, Heimrich B, and Schwemmle M (2013) Absence of a robust innate immune response in rat neurons facilitates persistent infection of Borna disease virus in neuronal tissue. *Cell Mol Life Sci* doi: 10.1007/s00018-013-1402-5.

- Moore EM, Nichol AD, Bernard SA, and Bellomo R (2011) Therapeutic hypothermia: benefits, mechanisms and potential clinical applications in neurological, cardiac and kidney injury. *Injury* **42**:843-854.
- Nairn AC and Palfrey HC (1987) Identification of the major Mr 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2. *J Biol Chem* **262**:17299-17303.
- Nordby HK and Nesbakken R (1984) The effect of high dose barbiturate decompression after severe head injury. A controlled clinical trial. *Acta Neurochir (Wien)* **72**:157-166.
- Oakhill JS, Scott JW, and Kemp BE (2012) AMPK functions as an adenylate charge-regulated protein kinase. *Trends Endocrinol Metab* **23**:125-132.
- Py BF, Boyce M, and Yuan J (2009) A critical role of eEF-2K in mediating autophagy in response to multiple cellular stresses. *Autophagy* **5**:393-396.
- Redpath NT and Proud CG (1993) Cyclic AMP-dependent protein kinase phosphorylates rabbit reticulocyte elongation factor-2 kinase and induces calcium-independent activity. *Biochem J* **293**:31-34.
- Roesslein M, Schibilsky D, Muller L, Goebel U, Schwer C, Humar M, Schmidt R, Geiger KK, Pahl HL, Pannen BH, and Loop T (2008) Thiopental protects human T lymphocytes from apoptosis in vitro via the expression of heat shock protein 70. *J Pharmacol Exp Ther* **325**:217-225.
- Rolfe DF and Brown GC (1997) Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol Rev* **77**:731-758.

- Ryazanov AG (1987) Ca²⁺/calmodulin-dependent phosphorylation of elongation factor 2. *FEBS Lett* **214**:331-334.
- Ryazanov AG, Shestakova EA, and Natapov PG (1988) Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation. *Nature* **334**:170-173.
- Sahni R and Weinberger J (2007) Management of intracerebral hemorrhage. *Vasc Health Risk Manag* **3**:701-709.
- Salminen A, Hyttinen JM, and Kaarniranta K (2011) AMP-activated protein kinase inhibits NF-kappaB signaling and inflammation: impact on healthspan and lifespan. *J Mol Med (Berl)* **89**:667-676.
- Schwer CI, Stoll P, Rospert S, Fitzke E, Schallner N, Burkle H, Schmidt R, and Humar M (2013) Carbon monoxide releasing molecule-2 CORM-2 represses global protein synthesis by inhibition of eukaryotic elongation factor eEF2. *Int J Biochem Cell Biol* **45**:201-212.
- Skellern GG, Stenlake JB, Williams WD, and McLarty DG (1974) Plasma concentrations of methimazole, a metabolite of carbimazole, in hyperthyroid patients. *Br J Clin Pharmacol* **1**:265-269.
- Stankowski JN and Gupta R (2011) Therapeutic targets for neuroprotection in acute ischemic stroke: lost in translation? *Antioxid Redox Signal* **14**:1841-1851.
- Steen PA and Michenfelder JD (1980) Mechanisms of barbiturate protection. *Anesthesiology* **53**:183-185.
- Terai K, Hiramoto Y, Masaki M, Sugiyama S, Kuroda T, Hori M, Kawase I, and Hirota H (2005) AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress. *Mol Cell Biol* **25**:9554-9575.

- Turcant A, Delhumeau A, Premel-Cabic A, Granry JC, Cottineau C, Six P, and Allain P (1985) Thiopental pharmacokinetics under conditions of long-term infusion. *Anesthesiology* **63**:50-54.
- Wang S, Xing Z, Vosler PS, Yin H, Li W, Zhang F, Signore AP, Stetler RA, Gao Y, and Chen J (2008) Cellular NAD replenishment confers marked neuroprotection against ischemic cell death: role of enhanced DNA repair. *Stroke* **39**:2587-2595.
- White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, and Krause GS (2000) Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J Neurol Sci* **179**:1-33.
- Won SJ, Kim DY, and Gwag BJ (2002) Cellular and molecular pathways of ischemic neuronal death. *J Biochem Mol Biol* **35**:67-86.
- Wong AK, Howie J, Petrie JR, and Lang CC (2009) AMP-activated protein kinase pathway: a potential therapeutic target in cardiometabolic disease. *Clin Sci (Lond)* **116**:607-620.
- Ying W, Wei G, Wang D, Wang Q, Tang X, Shi J, Zhang P, and Lu H (2007) Intranasal administration with NAD⁺ profoundly decreases brain injury in a rat model of transient focal ischemia. *Front Biosci* **12**:2728-2734.
- Yueh A and Schneider RJ (2000) Translation by ribosome shunting on adenovirus and hsp70 mRNAs facilitated by complementarity to 18S rRNA. *Genes Dev* **14**:414-421.
- Zhan RZ, Fujiwara N, Endoh H, Yamakura T, Taga K, Fukuda S, and Shimoji K (1998) Thiopental inhibits increases in [Ca²⁺]_i induced by membrane depolarization, NMDA receptor activation, and ischemia in rat hippocampal and cortical slices. *Anesthesiology* **89**:456-466.

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Zhang X, Gao R, Li J, Qi Y, Song X, Zhao L, Wang H, Pu Y, Xu K, and Li J (2010) A pharmacological activator of AMP-activated protein kinase protects hypoxic neurons in a concentration-dependent manner. *Neurochem Res* **35**:1281-1289.

Footnotes

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Figure Legends

Figure 1. *Carbimazole induces eEF2 phosphorylation.* Human neuronal SK-N-SH cells were treated with 10 μ M – 2 mM carbimazole for 6 h (A), or 0.5 mM carbimazole for 10 min to 24 h (B) before immunoblots were performed using a phospho-eEF2 threonine 56 antibody (upper blots) or an eEF2 antibody that detects endogenous levels of total eEF2, independently of phosphorylation (lower blots). The results shown are representative for four independent experiments.

Figure 2. *Exposure to carbimazole results in global translational inhibition.* Human neuronal SK-N-SH cells were exposed to 0.1 – 1 mM carbimazole or 5 μ g/ml cycloheximide (CHX) for 4 h and then pulsed with [35 S]methionine for an additional 2 h, before cell lysates were separated by SDS-PAGE and stained by Coomassie Brilliant Blue G-250. The amounts of *de novo* synthesized proteins were detected by autoradiography (A). (B), densitometric analysis of [35 S]methionine incorporation within SDS-PAGE gels. Values represent the mean \pm standard deviations of five separate experiments. Statistical differences were analyzed by performing one-way ANOVA followed by the Dunnett's multiple comparisons test. ***, $p < 0.001$ versus untreated [35 S]methionine labeled control cells. Total protein amounts within SDS-PAGE gels were visualized by Coomassie G-250 staining (C).

Figure 3. *Effects of carbimazole on eEF2K-dependent eEF2 phosphorylation and on cytoplasmic calcium levels.* In (A), eEF2K was inhibited by 100 μ M NH125 for 1 h before 0.5 mM carbimazole was added to SK-N-SH cells for an additional hour. Phosphorylation of eEF2 at threonine 56 (upper blot) or total cellular levels of eEF2 independently of phosphorylation (lower blot) were determined by immunoblotting. In (B), SK-N-SH cells were left untreated or were incubated with 0.5 mM carbimazole, 1 mM carbimazole, or 40 μ M thapsigargin in calcium containing (black bars) or calcium-free (white bars) growth

medium for 1 h. For the last 30 min of the experiment cells were loaded with 2 μ M fura-2 acetoxymethyl ester, before intracellular calcium complexes were excited at 340 nm and recorded at 510 nm. In (C/D), calcium-induced cytotoxicity was determined in SK-N-SH cells that were left untreated or were incubated in the presence of 0.5 mM or 1 mM carbimazole in calcium containing growth medium for 24 -72 h. Thapsigargin (40 μ M) was added for 1 h. Caspase-3 activity assays (C) and LDH-release assays (D) are shown. Values represent the mean \pm standard deviations of three independent experiments. Experimental groups were statistically analyzed by performing one-way ANOVA followed by the Dunnett's multiple comparisons test (B), unpaired t test (C/D, left graph), or two-way ANOVA followed by the Bonferroni's *post hoc* test (C/D, right graph). ***, $p < 0.001$ versus untreated control cells.

Figure 4. *Mechanism of eEF2 phosphorylation.* SK-N-SH cells were preincubated with 200 μ M camstatin (A), 200 μ M CGS 9343B (A), 1 mM cAMPS-Rp (C), 10 μ M PKI 14-22 amide (C), 1 μ M rapamycin (D), or 100 μ M dorsomorphin dihydrochloride (F) for 1 h, before eEF2 phosphorylation was induced with 0.5 mM carbimazole for an additional hour. Cell lysates were analyzed by immunoblotting using an anti-human phospho-eEF2 threonine 56 antibody (upper blots) or an anti-eEF2 antibody that detects endogenous levels of total eEF2 independently of phosphorylation (lower blots). In (B), intracellular cAMP content was determined by a competitive cAMP immunoassay using SK-N-SH cells that were left untreated or were incubated with 0.5 mM or 1 mM carbimazole for 15 min to 6 h before cell lysis. Values represent the mean \pm standard deviations of three independent experiments. Statistical differences were analyzed by performing two-way ANOVA followed by the Bonferroni's *post hoc* test. **, $p < 0.01$ for cells treated with 1 mM carbimazole versus the untreated control. In (E), immunoblots were performed using SK-N-SH cells that were incubated with 0.5 mM carbimazole for 10 min to 6 h to determine autophosphorylation of AMPK at threonine 172 (upper blot) or endogenous levels of total AMPK, independently of

phosphorylation (lower blot). The immunoblots shown are representative for four independent experiments.

Figure 5. *Effect of carbimazole on hypoxic neuronal cell injury.* Cellular damage was induced in human neuronal SK-N-SH cells by oxygen deprivation (closed symbols) in an atmosphere containing 5% CO₂, 95% N₂ and measured by an LDH release assay. Control cells were cultured in 5% CO₂, 21% O₂, and 74% N₂ (open symbols). In (A), cell damage was measured in the presence (squares) or absence (rhombi) of fetal calf serum. In (B), 0.5 mM carbimazole was added to serum containing growth medium (triangles). Values represent the mean ± standard deviations of four separate experiments. Experimental groups were statistically analyzed by performing two-way ANOVA followed by the Bonferroni's *post hoc* test. Statistically significant differences within groups shown for (A) are: serum treated oxygen deprived SK-N-SH cells versus serum treated normoxic control cells (***, $p < 0.001$). Statistically significant differences within groups shown for (B) are: oxygen deprived SK-N-SH cells versus normoxic control cells (***, $p < 0.001$); versus oxygen-deprived cells treated with 0.5 mM carbimazole (###, $p < 0.001$); versus normoxic control cells in the presence of 0.5 mM carbimazole (***, $p < 0.001$); and normoxic control cells versus oxygen-deprived cells in the presence of 0.5 mM carbimazole (¶, $p < 0.01$).

Figure 6. *Effect of carbimazole on intracellular ATP content.* SK-N-SH cells were cultured in an oxygen-free atmosphere containing 5% CO₂, 95% N₂ for 12 to 72 h in the presence (closed circles) or absence (closed squares) of 0.5 mM carbimazole. Cells, cultured in a normoxic atmosphere containing 5% CO₂, 21% O₂, and 74% N₂ (open squares) and normoxic cells cultured in the presence of 0.5 mM carbimazole (open circles) served as controls. ATP content of cells was measured in lysates by an ATP-driven luciferase assay. Determined relative light units (RLU) were normalized to protein content and represent the means ±

standard deviations of four independent experiments. Experimental groups were statistically evaluated by performing two-way ANOVA followed by the Bonferroni's *post hoc* test. Statistically significant differences within groups are: oxygen deprived SK-N-SH cells versus normoxic control cells (***, $p < 0.001$); versus oxygen-deprived cells treated with 0.5 mM carbimazole (###, $p < 0.001$); versus normoxic control cells in the presence of 0.5 mM carbimazole (***, $p < 0.001$); and normoxic control cells versus oxygen-deprived cells in the presence of 0.5 mM carbimazole (###, $p < 0.001$).

Figure 7. *Hypoxia induces eEF2 and AMPK phosphorylation.* Human neuronal SK-N-SH cells were exposed to hypoxia for 0 – 72 h and analyzed by immunoblotting with an anti-human phospho-eEF2 threonine 56 or an anti-human phospho-AMPK threonine 172 antibody. The immunoblots were normalized using antibodies that detected endogenous levels of total eEF2 or AMPK independently of phosphorylation. The results shown are representative for three independent experiments.

Figure 8. *Effect of cycloheximide and anisomycin on hypoxic neuronal cell injury and intracellular ATP content.* (A/B), measurement of hypoxic injury by an LDH release assay. (C/D), measurement of relative ATP content by an ATP-driven luciferase assay. Cells were cultured in a hypoxic atmosphere containing 5% CO₂, 95% N₂ (closed squares) and treated with 5 µg/ml cycloheximide (closed triangles), 1 µg/ml anisomycin (closed rhombi), or 10 ng/ml anisomycin (closed circles) for up to 72 h. Control cells were cultured in 5% CO₂, 21% O₂, and 74% N₂ (open squares) and treated with 5 µg/ml cycloheximide (open triangles), 1 µg/ml anisomycin (open rhombi), or 10 ng/ml anisomycin (open circles). Values represent the mean ± standard deviations of four separate experiments. Experimental groups were statistically analyzed by performing two-way ANOVA followed by the Bonferroni's *post hoc* test. Statistically significant differences within groups are: oxygen deprived SK-N-SH cells

versus normoxic control cells (***, $p < 0.001$); versus normoxic cells in the presence of 1 $\mu\text{g/ml}$ cycloheximide (^{yyy}, $p < 0.001$); versus normoxic cells in the presence of 1 $\mu\text{g/ml}$ anisomycin (^{fff}, $p < 0.001$); versus normoxic cells in the presence of 10 ng/ml anisomycin (^{sss}, $p < 0.001$); versus hypoxic cells in the presence of 1 $\mu\text{g/ml}$ cycloheximide (^{###}, $p < 0.001$); versus hypoxic cells in the presence of 1 $\mu\text{g/ml}$ anisomycin (^{HH}, $p < 0.01$; ^{HHH}, $p < 0.001$); versus hypoxic cells in the presence of 10 ng/ml anisomycin (^{fff}, $p < 0.001$). The statistically significant differences within groups of normoxic control cells versus normoxic cells in the presence of 1 $\mu\text{g/ml}$ cycloheximide (^ψ, $p < 0.05$); versus normoxic cells in the presence of 1 $\mu\text{g/ml}$ anisomycin (^{ccc}, $p < 0.001$); versus hypoxic cells in the presence of 1 $\mu\text{g/ml}$ anisomycin (^{TTT}, $p < 0.001$); and versus hypoxic cells in the presence of 10 ng/ml anisomycin (^β, $p < 0.05$) are also shown.

Figure 9. *Carbimazole inhibits protein synthesis, ameliorates hypoxic damage, and maintains energy balance during oxygen deprivation in primary cortical neurons.* In (A), cortical neurons were treated with 10 μM - 2 mM carbimazole for 6 h and analyzed for phosphorylation of eEF2 and AMPK by immunoblotting. In (B), cortical neurons were left untreated or were exposed to 0.1 – 1 mM carbimazole for 6 h and then pulse labeled with 200 μCi of [³⁵S]methionine for an additional 2 h. Cellular lysates were separated by 10% SDS-PAGE and the amount of newly synthesized protein was detected by autoradiography on dried electrophoresis gels. In (C), cortical neurons were exposed to hypoxia for 48 h in the presence or absence of 0.5 mM carbimazole. Cellular damage was determined by an LDH-release assay. In (D), cortical neurons were exposed to hypoxia for 0 – 72 h or 40 μM thapsigargin for 1 h and analyzed by immunoblotting for phosphorylation of eEF2, pro-caspase 3 cleavage, and PARP cleavage. The immunoblots were normalized using an antibody that detected α -tubulin. In (E), cortical neurons were exposed to hypoxia for 48 h in the presence or absence of 0.5 mM carbimazole. The relative intracellular ATP-content was

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measured by an ATP-driven luciferase assay. Values represent the mean \pm standard deviations of three independent experiments. Statistical evaluation of experimental groups was performed by one-way ANOVA followed by the Bonferroni's *post hoc* test. The statistically significant difference of oxygen-deprived cortical neurons in the presence or absence of carbimazole is shown (***, $p < 0.001$).

Figure 1

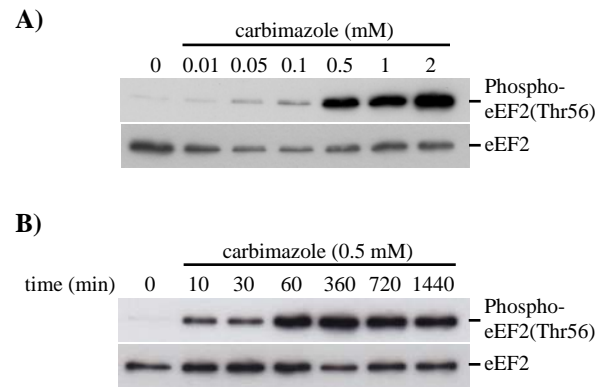


Figure 2

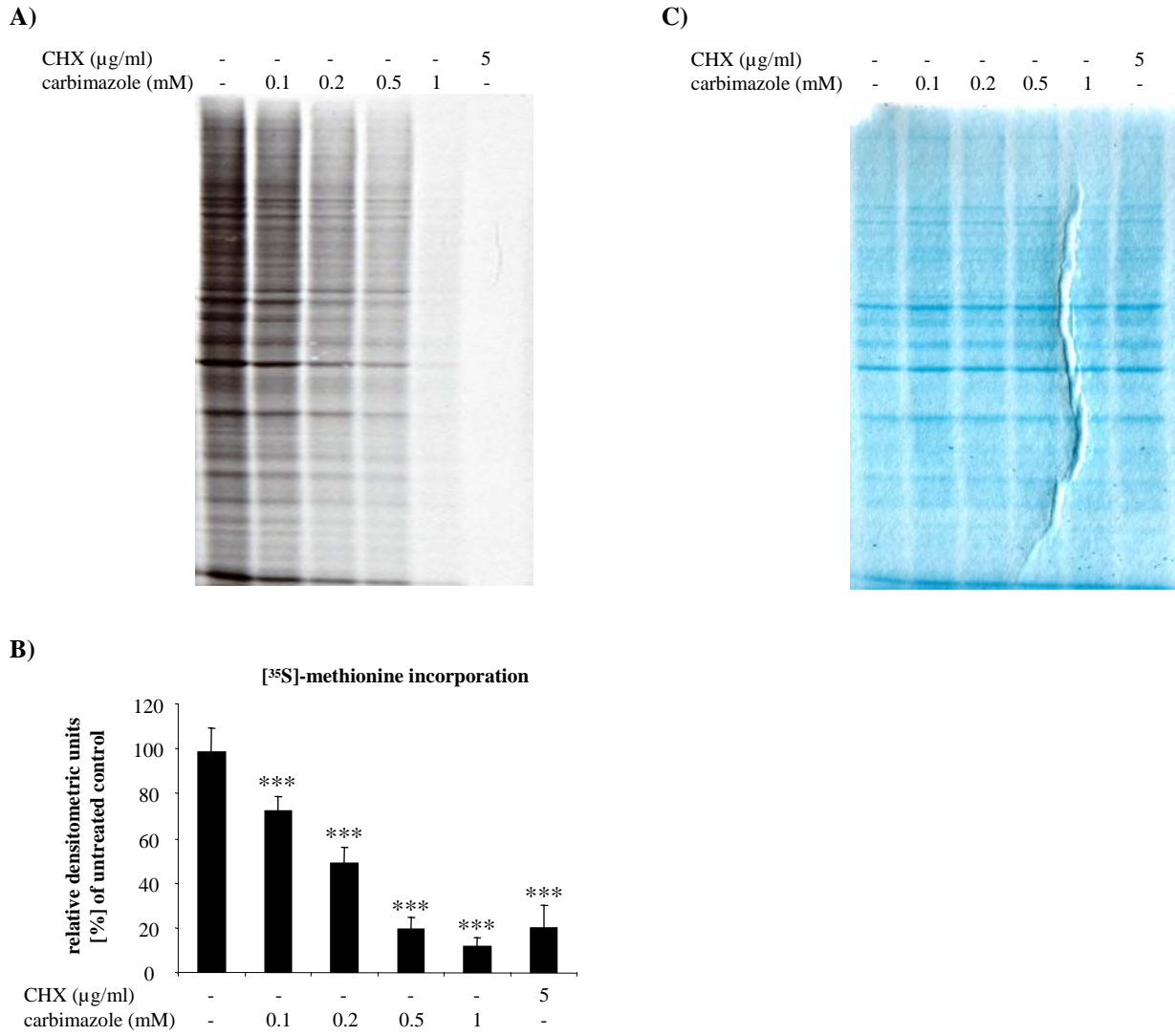


Figure 3

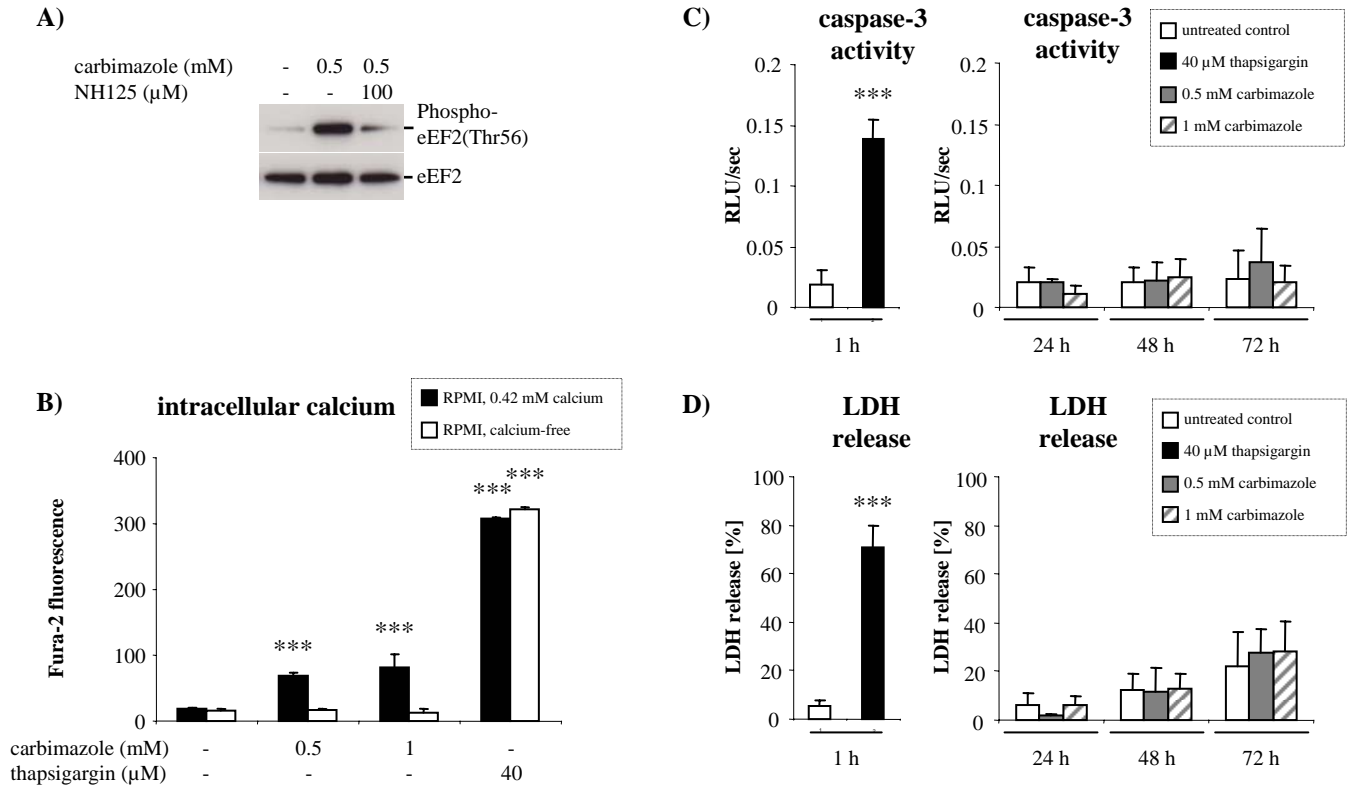


Figure 4

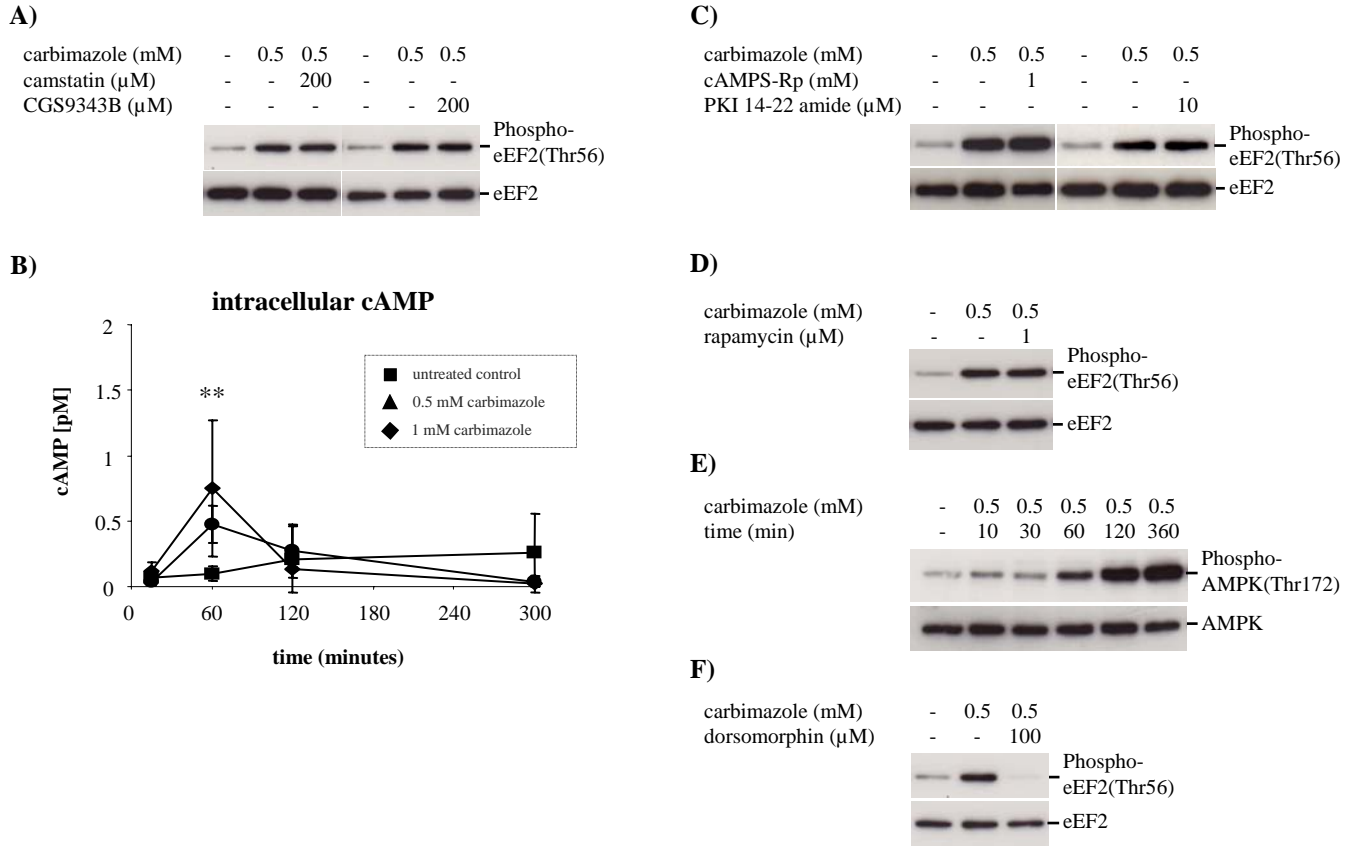


Figure 5

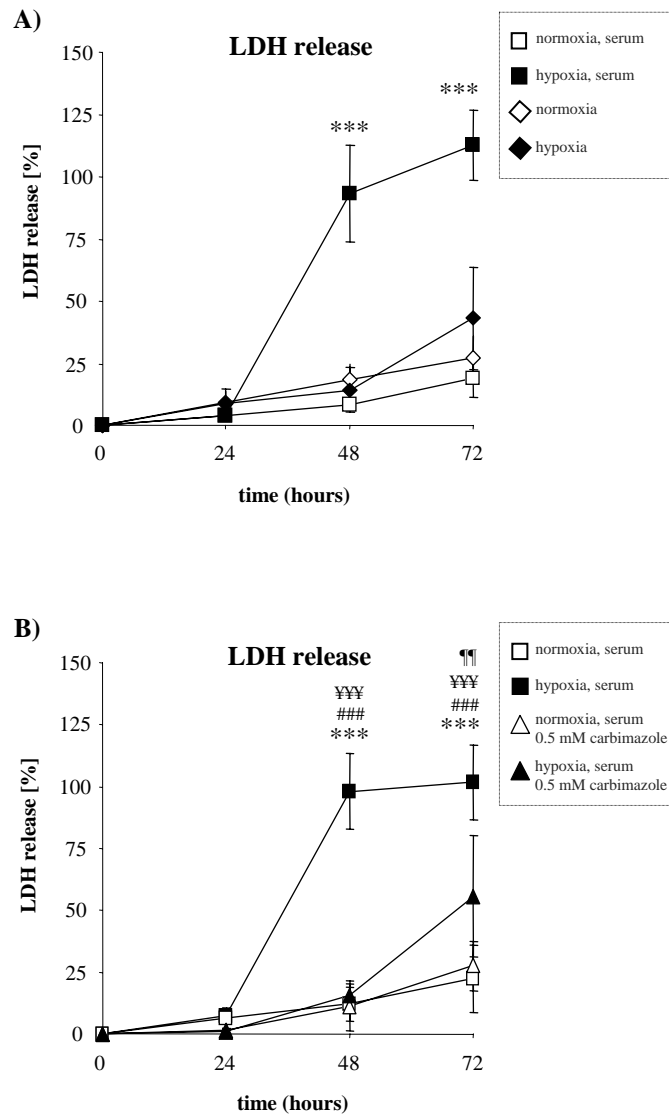


Figure 6

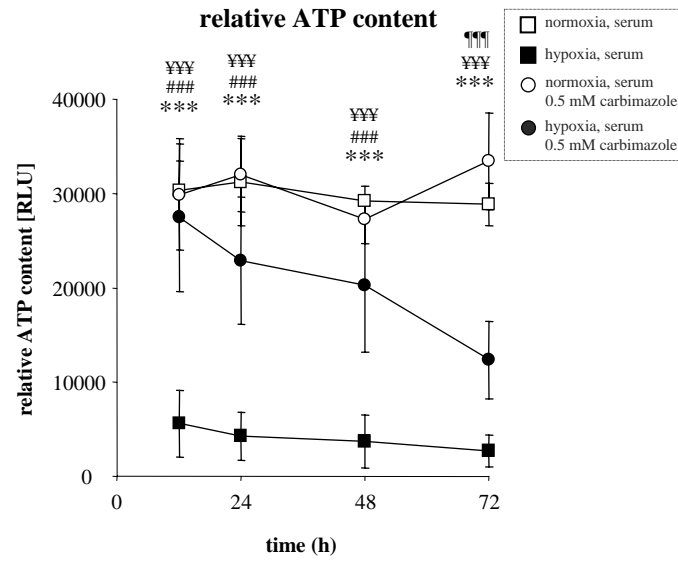
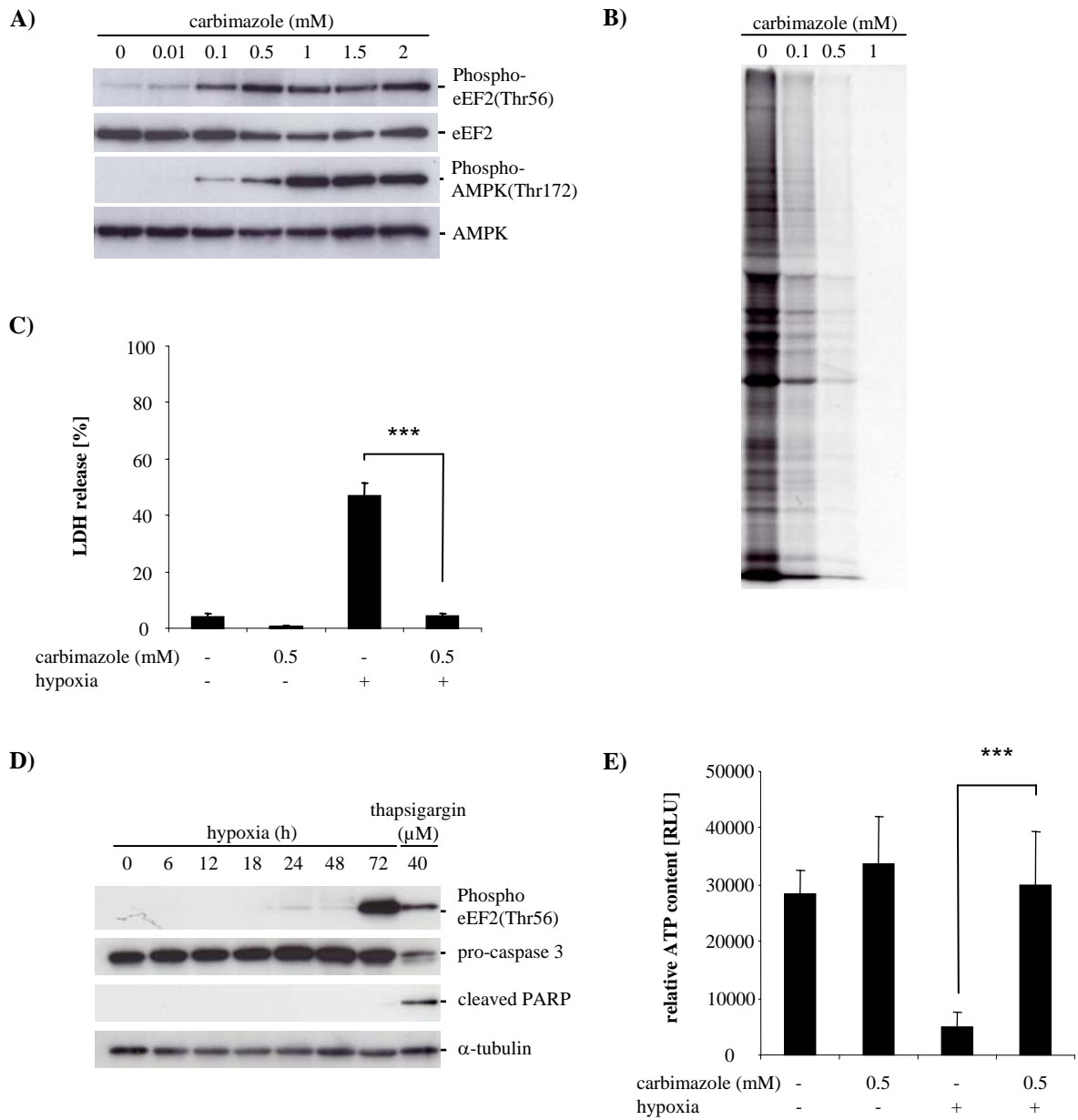


Figure 9



SUPPLEMENTAL DATA

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Carbimazole is a translational inhibitor and protects from neuronal hypoxic damage

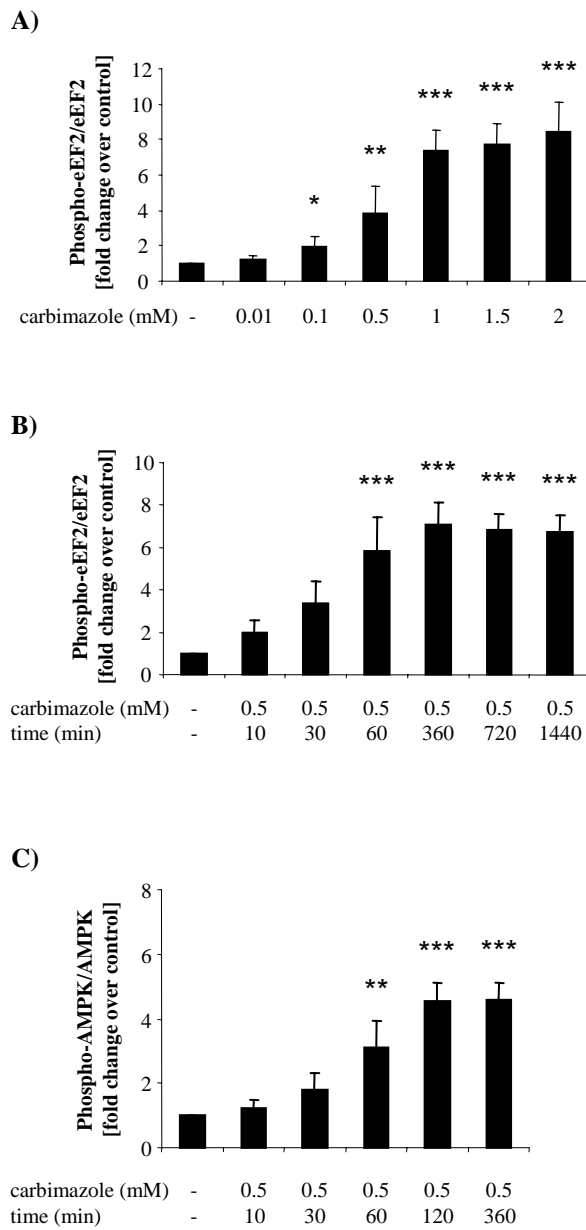
Cornelius Lehane, Timo Guelzow, Simone Zenker, Anika Erxleben, Christian I. Schwer,
Bernd Heimrich, Hartmut Buerkle and Matjaz Humar

Department of Anesthesiology and Critical Care Medicine, University Medical Center
Freiburg, Freiburg, Germany (C.L., C.I.S., H.B., M.H.);

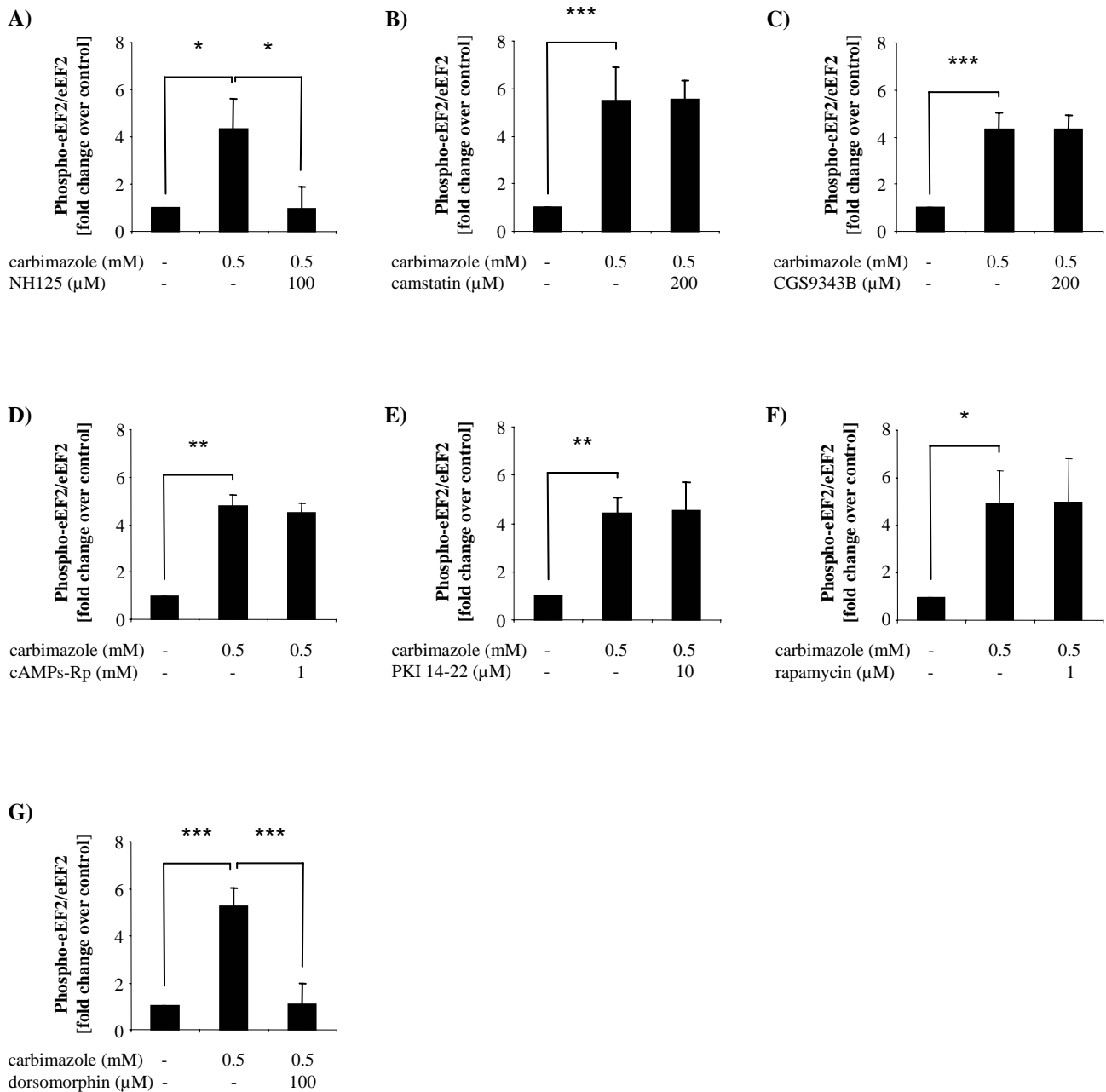
Department of General Neurosurgery, Cellular Neurophysiology, University Medical Center
Freiburg, Freiburg, Germany (T.G.);

Department of Anatomy and Cell Biology, Albert-Ludwigs-University of Freiburg, Freiburg,
Germany (S.Z., B.H.);

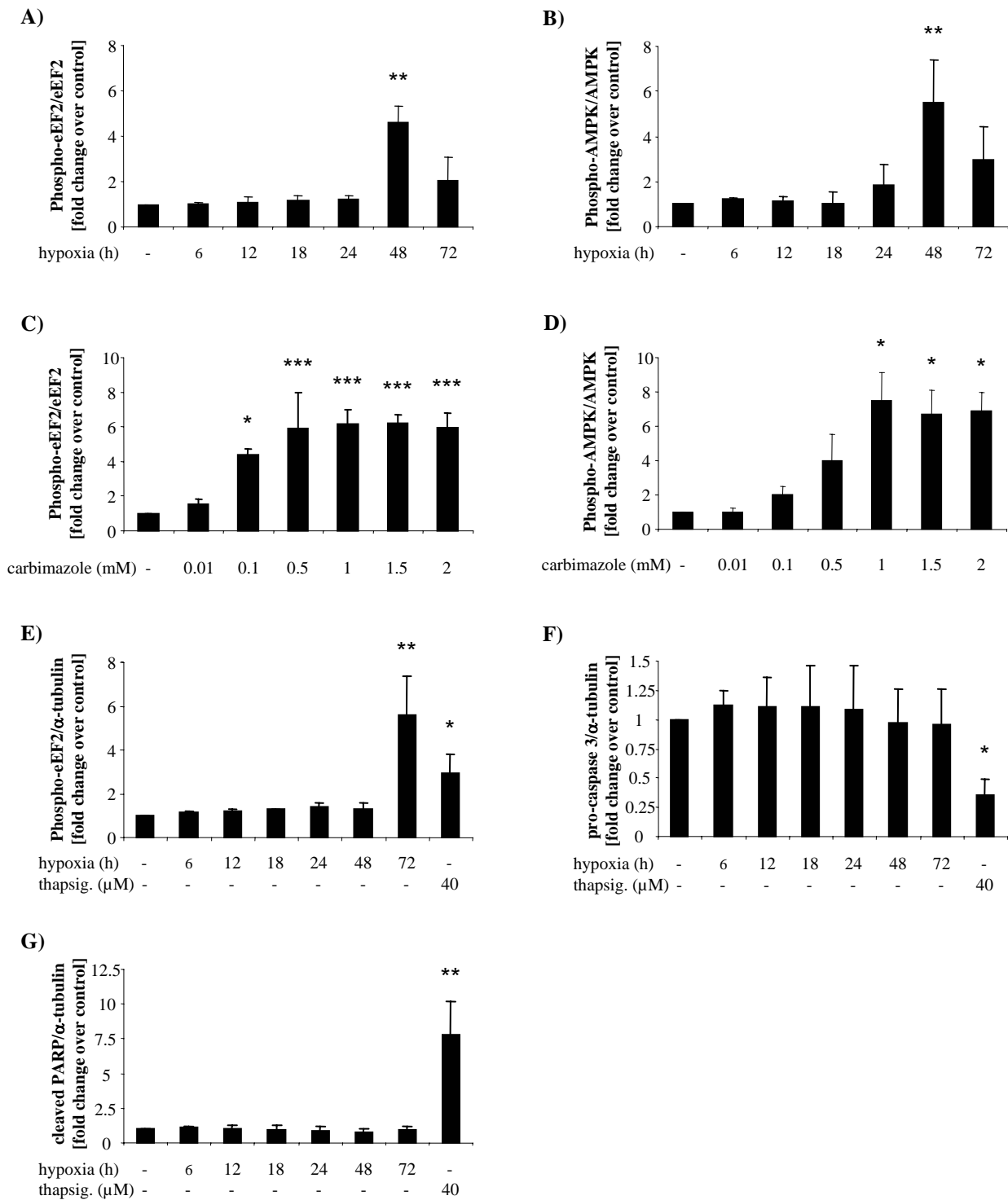
Pharmaceutical Bioinformatics, Institute of Pharmaceutical Sciences, Albert-Ludwigs-
University of Freiburg, Freiburg, Germany (A.E.).



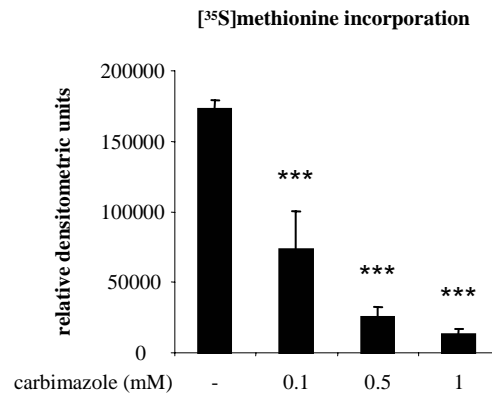
Supplemental Figure 1. Densitometric analysis of immunoblots depicted in Fig. 1A (**A**), Fig. 1B (**B**), and Fig. 4E (**C**). The values are the means \pm standard deviations. Statistical differences were analyzed by performing one-way ANOVA followed by the Bonferroni's *post hoc* test. *, $p < 0.05$ (**, $p < 0.01$; ***, $p < 0.001$) versus non stimulated control cells (bar 1).



Supplemental Figure 2. Densitometric analysis of immunoblots depicted in Fig. 3A (A), Fig. 4A (B/C), Fig. 4C (D/E), Fig. 4D (F), and Fig. 4F (G). The values are the means \pm standard deviations. Statistical differences were analyzed by performing one-way ANOVA followed by the Bonferroni's *post hoc* test. *, $p < 0.05$ (**, $p < 0.01$; ***, $p < 0.001$) versus carbimazole treated cells (bar 2).



Supplemental Figure 3. Densitometric analysis of immunoblots depicted in Fig. 7 (A/B), Fig. 9A (C/D), and Fig. 9D (E-G). The values are the means \pm standard deviations. Statistical differences were analyzed by performing one-way ANOVA followed by the Bonferroni's *post hoc* test. *, $p < 0.05$ (**, $p < 0.01$; ***, $p < 0.001$) versus non stimulated control cells (bar 1).



Supplemental Figure 4. Densitometric analysis of [³⁵S]methionine incorporation within SDS-PAGE gels, depicted in Fig. 9B. Values represent the means \pm standard deviations of four separate experiments. Statistical differences were analyzed by performing one-way ANOVA followed by the Bonferroni's *post hoc* test. ***, $p < 0.001$ versus untreated [³⁵S]methionine labelled control cells (bar 1).