Chronic stress targets posttranscriptional mechanisms to rapidly upregulate α1C-subunit of CaV1.2b calcium channels in colonic smooth muscle cells

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Li Q, Sarna SK. Chronic stress targets posttranscriptional mechanisms to rapidly upregulate α1C-subunit of CaV1.2b calcium channels in colonic smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 300: G154–G163, 2011. First published November 4, 2010; doi:10.1152/ajpgi.00393.2010.—Chronic stress elevates plasma norepinephrine, which enhances expression of the α1C-subunit of CaV1.2b channels in colonic smooth muscle cells within 1 h. Transcriptional upregulation usually does not explain such rapid protein synthesis. We investigated whether chronic stress-induced release of norepinephrine utilizes posttranscriptional mechanisms to enhance the α1C-subunit. We performed experiments on colonic circular smooth muscle strips and in conscious rats, using a 9-day chronic intermittent stress protocol. Incubation of rat colonic muscularis externa with norepinephrine enhanced α1C-protein expression within 45 min, without a concomitant increase in α1C mRNA, indicating posttranscriptional regulation of α1C-protein by norepinephrine. We found that norepinephrine activates the PI3K/Akt/GSK-3β pathway to concurrently enhance α1C-protein translation and block its polyubiquitination and proteasomal degradation. Incubation of colonic muscularis externa with norepinephrine or LiCl, which inhibits GSK-3β, enhanced p-GSK-3β and α1C-protein time dependently. Using enrichment of phosphoproteins and ubiquitinated proteins, we found that both norepinephrine and LiCl decrease α1C phosphorylation and polyubiquitination. Concurrently, they suppress eIF2α (Ser51) phosphorylation and 4E-BPI expression, which stimulates gene-specific translation. The antagonism of two upstream kinases, PI3K and Akt, inhibits the induction of α1C-protein by norepinephrine. Cytomedrol (β2-AR antagonist) partially suppresses norepinephrine-induced α1C-protein expression, whereas phenolamine and prazosin (α-AR and α1-AR antagonist, respectively) have no significant effect. Experiments in conscious animals showed that chronic stress activates the PI3K/Akt/GSK-3β signaling. We conclude that norepinephrine released by chronic stress rapidly enhances the protein expression of α1C-subunit of CaV1.2b channels by concurrently suppressing its degradation and enhancing translation of existing transcripts to maintain homeostasis.

irritable bowel syndrome; GSK-3; posttranslational regulation; Wnt signaling

STRESS RESPONSE IS ORGANISMS’ defense against real or perceived threats. All psychological stress responses begin with the release of corticotrophin-releasing hormone and arginine vasopressin in the paraventricular nucleus of the hypothalamus (37), which stimulate the neuroendocrine axis to release a group of hormones and neurotransmitters systemically and in select organ tissues (5). The activation of the sympathetic nerves immediately releases norepinephrine at their junctions with the target cells, such as cardiomyocytes, vascular smooth muscle cells and skeletal muscle cells. The stimulation of the adrenal medulla elevates the plasma levels of norepinephrine and epinephrine. The release of catecholamines from the adrenal medulla is significantly slower than that of norepinephrine from sympathetic nerve endings (6, 8, 29, 36).

The adaptive response to acute stress is swift to maintain homeostasis. The mechanisms by which acute stress rapidly primes the cardiovascular and skeletal muscle cells are well understood (25, 26, 40). The release of norepinephrine from the sympathetic neurons induces second messenger-activated protein kinase phosphorylation of CaV1.2 (L-type) calcium channels to instantaneously increase inward calcium current and enhance channel activity. Calcium influx through CaV1.2 channels is an essential early step in the contraction of cardiomyocytes, vascular and gut smooth muscle, and skeletal muscle cells. However, this abrupt and robust response is not sustainable for long periods and it subsides soon after the threat recedes.

If stress persists for long duration or if it occurs frequently, it becomes maladaptive, in which case the stress mediators cause organ dysfunction, such as cardiac hypertrophy and failure (2). In gut, chronic stress is a major factor in the exaggeration or relapse of the symptoms of irritable bowel syndrome (3, 38) and inflammatory bowel disease (21). We reported recently that norepinephrine release from the adrenal medulla mediates the maladaptive effects of heterotypic intermittent chronic stress (HeICS) (8). Specifically, it enhances protein expression of the pore-forming α1C-subunit of CaV1.2b channels in colonic circular smooth muscle cells, which enhances calcium influx, reactivity to acetylcholine, and colonic transit.

We do not know the cellular mechanisms by which norepinephrine enhances expression of the α1C-subunit. The plasma norepinephrine increases significantly within 4 h, peaks at ~8 h after the last stressor of the 9-day HeICS protocol, and returns to baseline by 24 h (8). The increase and decrease in expression of the α1C-subunit follow very closely the increase and decrease in plasma norepinephrine following chronic stress, which makes transcriptional upregulation of the α1C-subunit unlikely. The transcriptional upregulation of the α1C-subunit takes ~6 h (33). We tested the hypothesis that norepinephrine stimulates the phosphatidylinositol 3-kinase (PI3K)/Akt/GSK-3β signaling pathway to prevent phosphorylation of the α1C-subunit protein followed by its polyubiquitination and proteasomal degradation. At the same time, pGSK-3β stimulates translation of the α1C transcripts to rapidly (in less than 1 h) enhance expression of this protein. We performed the
experiments on conscious rats and on rat colonic muscularis externa tissues.

MATERIALS AND METHODS

Reagents. Norepinephrine, calphostin C, Akt inhibitor Akti-1/2, and KN-93 were purchased from CalBiochem (Gibbstown, NJ); actinomycin D, LY294002, H89, phenolamine, prazosin, propranolol, cyanopindolol hemifumarate, and CL316243 from Sigma Aldrich (St. Louis, MO); SB239063, PD98059, and SP600125 from Tocris Bioscience (Ellisville, MO); and 1-[(14C)U]phenylalanine (>450 mCi/mmoll, Soluene 350, and Ultima Gold XR liquid scintillation cocktail from PerkinElmer (Waltham, MA).

Animals. Sprague-Dawley rats were housed in controlled temperature (22°C) and 12-h light-dark cycle. The animals were euthanized in a CO2 chamber. The animal use was approved by the IACUC at the University of Texas Medical Branch at Galveston.

HeICS protocol. We used a 9-day HeICS protocol comprised of daily application of one of the three stressors: water avoidance stress (60 min), forced swimming stress (20 min), and cold-restraint stress (45 min) (8). Sham-stressed rats served as controls. Animals were euthanized 8 h after the last stress session. Muscularis externa tissues were collected, snap frozen in liquid nitrogen, and stored at −80°C for molecular studies.

Preparation of rat colonic muscle strips. Freshly obtained full-thickness rat colonic tissues were immersed in warm, carbenogated Krebs solution with 5% O2-95% CO2 mix (8). The mucosal/submucosal layers were removed under magnifying glass. Muscle strips (2 mm × 10 mm) were cut along the circumferential axis and placed in high-glucose DMEM containing 10% FBS and appropriate chemicals.

Coimmunoprecipitation and immunoblotting. Coimmunoprecipitation and Western blotting were performed as described previously (27). Antibodies are as follows: anti-Cα1.2 rabbit polyclonal (Alomone Labs, Jerusalem, Israel); anti-phospho-NF-κB p65 (Ser536) rabbit antibody, anti-β-catenin rabbit polyclonal antibody, anti-actin rabbit (Ser473) rabbit polyclonal, anti-phospho-eukaryotic translation initiation factor 2α (eIF2α) Ser51 rabbit polyclonal, anti-4E-BP1 rabbit polyclonal (Cell Signaling, Danvers, MA); anti-phospho-GSK-3α/β (S21/S9) rabbit polyclonal (R&D Systems, Minneapolis, MN); anti-histone H3 COOH-terminal rabbit polyclonal (Active Motif, Carlsbad, CA); anti-GSK-3β rabbit polyclonal (Santa Cruz, CA); anti-β-actin mouse monoclonal (Sigma-Aldrich).

Real-time RT-PCR. Total RNA was extracted by use of RNeasy mini kit (Qiagen, Valencia, CA). One microgram of total RNA was reverse transcribed by use of SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Quantification of α1C gene expression by real-time PCR was performed with a StepOne Plus thermal system by binding DNA at the transcription initiation complex to prevent elongation by RNA polymerase (34). These findings suggest posttranscriptional upregulation of α1C-subunit by norepinephrine.

RESULTS

Norepinephrine rapidly elevates α1C-protein, but not mRNA of the α1C-subunit. The incubation of freshly prepared colonic muscle strips with 1 μM norepinephrine in complete culture medium DMEM significantly enhanced protein expression of the α1C-subunit of Cα1.2b channels within 45 min (Fig. 1, A and B). The increase persisted for at least 12 h. Therefore, the protein expression declined to basal level by 24 h. In addition, the washout of norepinephrine from the medium after 3 h degraded the enhanced protein to basal level within 1.5 h (Fig. 1C, top bands). Quantitative RT-PCR showed that incubation with norepinephrine for up to 48 h had no significant effect on expression of the α1C-subunit mRNA (Fig. 1D). The inclusion of 1 μg/ml actinomycin D in the medium had no significant impact on the increase of α1C-subunit by norepinephrine at 3 h (data not shown). Actinomycin D blocks gene transcription by binding DNA at the transcription initiation complex to prevent elongation by RNA polymerase (34). These findings suggested posttranscriptional upregulation of α1C-subunit by norepinephrine.
GSK-3β, which phosphorylates serine and threonine residues in some of its substrates, such as β-catenin, sets the stage for their polyubiquitination followed by proteasomal degradation (39). We tested the hypothesis that the α1C-subunit of Ca,1.2b channels and the eIF2α are substrates of GSK-3β. Norepinephrine activates PI3K/Akt resulting in inactivation of GSK-3β, which decreases phosphorylation of the α1C-subunit and eIF2α, leading to protein...

Fig. 1. Norepinephrine (NE) elevates α1C-protein but not mRNA of the α1C-subunit in rat colonic muscularis externa. A and B: immunoblotting analysis of α1C-protein expression after 1 µM NE treatment. β-Actin served as control. N = 4, *P < 0.05. C: washout of NE after 3-h incubation decreases the expression of α1C-protein and the phosphorylations of Akt and GSK-3β to basal level in less than 1.5 h. D: real-time RT-PCR analysis of α1C mRNA level after treatment with 1 µM NE. 18S was used as internal control. N = 7.

Fig. 2. A: LiCl enhances α1C-protein expression within 1-h of treatment; 20 mM NaCl served as control (N = 4). B: LiCl has no significant effect on α1C mRNA expression detected by real-time RT-PCR with 18S as internal control (N = 6). C and D: LiCl and NE time dependently increase GSK-3β phosphorylation (N = 3). E: NE induces β-catenin protein expression (N = 3). *P < 0.05 vs. control at time 0 in all bar graphs.
stabilization and rapid translational synthesis of de novo αC-subunit, respectively.

We incubated colonic muscle strips with 20 mM LiCl, which inactivates GSK-3β by phosphorylation and used 20 mM NaCl as control. The accumulation of αC-protein significantly increased in response to LiCl at 1-, 3-, and 6-h time points; it started to decline thereafter (Fig. 2A). LiCl did not change mRNA expression of the αC-subunit (Fig. 2B). These data are similar to those in response to norepinephrine (Fig. 1, A and D). The phosphorylation of GSK-3β increased significantly within 1 h, indicating that LiCl inhibits the activities of GSK-3β (Fig. 2C). We found that norepinephrine also enhances pGSK-3β (Fig. 2D), suggesting that norepinephrine inactivates GSK-3β by its phosphorylation. The phosphorylation of GSK-3β in response to LiCl persisted for the entire incubation period of 24 h, but its phosphorylation with norepinephrine peaked at ~12 h and it declined thereafter, indicating a self-limiting mechanism. Note that the phosphorylation of GSK-3β also declined rapidly when norepinephrine was washed out after 3-h incubation (Fig. 1C).

GSK-3β inactivation is well known to accumulate β-catenin. We found that norepinephrine treatment of the colonic muscularis externa also accumulates β-catenin, starting as early as 15 min after treatment with norepinephrine (Fig. 2E).

Norepinephrine and LiCl suppress phosphorylation and polyubiquitination of αC. We investigated whether the inactivation of GSK-3β by norepinephrine or LiCl decreases the phosphorylation of αC. We treated colonic muscle strips with 1 μM norepinephrine or 20 mM LiCl for 1 and 3 h and prepared whole cell lysates. We enriched phosphoproteins with a phosphoprotein enrichment kit (Pierce) and subjected the pre-enriched phosphoproteins for ubiquitination followed by proteasomal degradation (39). To confirm that αC phosphorylation is catalyzed by GSK-3β, we incubated rat colonic muscularis externa with 1 μM norepinephrine or 20 mM LiCl for 1 and 3 h and prepared whole cell lysates with RIPA buffer. Polyubiquitinated proteins were then precipitated by use of a ubiquitin enrichment kit (Pierce) and assayed by immunoblotting. (Ub)n-αC phosphorylation is catalyzed by GSK-3β, a unique kinase that is constitutively active.

In many cases, GSK-3β phosphorylates and earmarks target proteins for ubiquitination followed by proteasomal degradation (39). To confirm that αC phosphorylation is essential for its polyubiquitination, we incubated rat colonic muscularis externa with 1 μM norepinephrine or 20 mM LiCl for 1 and 3 h and prepared whole cell lysates with RIPA buffer. Polyubiquitinated proteins were then precipitated by use of a ubiquitin enrichment kit (Pierce) and assayed by immunoblotting. (Ub)n-αC phosphorylation and polyubiquitination in rat colonic muscularis externa (N = 3). P-p65 and (Ub)n-H3 were used as loading controls for phosphoproteins and polyubiquitinated proteins, respectively. *P < 0.05 vs. controls. B: GSK-3β coimmunoprecipitates with αC in rat colonic muscularis externa (N = 3). IB, immunoblot; IP, immunoprecipitation. NE (C) and LiCl (D) rapidly increase Akt phosphorylation (N = 3). E: phosphatidylinositol 3-kinase inhibitor LY294002 suppresses NE-induced αC accumulation. Akt activation, and GSK-3β phosphorylation (N = 3). F: αC, pAkt, and pGSK-3β induction by NE is abrogated by Akt inhibitor Akti-1/2.
phosphorylation is essential for its polyubiquitination and proteasomal degradation. Polyubiquitinated histone H3 ([Ub]5-H3) was also probed and served as control.

We confirmed that α1C is a substrate of GSK-3β by coimmunoprecipitation using anti-α1C antibody to examine GSK-3β/α1C interaction. GSK-3β indeed coimmunoprecipitated with α1C (Fig. 3B), indicating that GSK-3β associates with α1C and α1C is one of the target proteins of GSK-3β.

Norepinephrine activates PI3K/Akt in rat colonic circular smooth muscle cells. GSK-3β is one of the known targets of PI3K/Akt pathway. Therefore, we investigated whether this pathway mediates GSK-3β phosphorylation in response to norepinephrine. We incubated rat colonic circular muscle strips with 1 μM norepinephrine or 20 mM LiCl for 0, 1, 3, 6, 12, and 24 h and assayed pAkt by immunoblotting analysis. Both norepinephrine and LiCl elevated pAkt within 1 h (Fig. 3, C and D); the pAkt level in response to norepinephrine peaked in ~6 h and declined thereafter. Note that the washout of norepinephrine after 3-h incubation rapidly decreased pAkt (Fig. 1E).

Next, we treated colonic muscularis externa with either LY294002 (PI3K inhibitor; 0.5 and 1 μM) or Akti-1/2 (0.05, 0.1, and 0.5 μM) for 30 min, followed by 6-h incubation with 1 μM norepinephrine to investigate whether PI3K and Akt mediate norepinephrine-induced upregulation of α1C. Both concentrations of LY294002 blocked the increase in α1C by norepinephrine (Fig. 3E). Only the two higher concentrations of Akti-1/2 blocked the enhancement of α1C by norepinephrine (Fig. 3F). Both antagonists concurrently inhibited the phosphorylation of Akt and GSK-3β at their effective concentrations.

AR subtypes that activate the PI3K/Akt/GSK-3β pathway. Preincubation of colonic muscle strips with the α-adrenergic receptor (AR) antagonist phentolamine showed no significant effect on the elevation of α1C by norepinephrine (Fig. 4A). Prazosin, an α1-AR antagonist, also had no significant effect on norepinephrine-induced α1C-subunit expression (Fig. 4B). The β1/2-AR antagonist propranolol (Fig. 4C) and β3-AR antagonist cyanopindolol (Fig. 4D) significantly inhibited the increase of α1C-subunit by norepinephrine. The β1/2-AR antagonist propranolol had a less robust effect. The α-AR and β1/2-AR antagonists showed interrat variability in their effects among animals in each group. However, the inhibitory effects of β3-AR antagonist were consistent among all rats in this group. In support of this observation, CL316243, a β3-AR agonist, markedly elevated the protein level of α1C-subunit (Fig. 4E).

The activation of adrenergic receptors accumulates cAMP in the cytoplasm and elevates intracellular calcium that activates PKA, PKC, calcium-calmodulin kinase II (CaMKII), and mitogen-activated protein kinases (MAPKs) (2). We preincubated the colonic muscularis externa tissues with the antagonists of these kinases to examine whether they contribute to the accumulation of α1C by norepinephrine. We found that none of the
antagonists of PKC (calphostin C), PKA (H-89), CaMKII (KN-93), ERK1/2 (PD98059), p38 (SB239063), or JNK (SP600125) had a significant effect on the accumulation of α1C by norepinephrine (Fig. 5, A and B).

Norepinephrine and LiCl suppress eIF2α (Ser51) phosphorylation and 4E-BP1 protein expression through Akt/GSK-3β pathway. Translation repressor protein 4E-BP1 inhibits cap-dependent translation by binding to the eIF-4E translation initiation factor (31). The phosphorylation of eIF2α (Ser51) is a well documented mechanism of downregulating protein synthesis under a variety of stress conditions (11). Both 4E-BP1 and eIF2α regulate global translation and gene-specific mRNA translation (12). We tested the hypothesis that norepinephrine and LiCl enhance α1C mRNA translation by suppressing 4E-BP1 and p-eIF2α (Ser51) in rat colonic muscle strips. We found that norepinephrine treatment significantly suppresses eIF2α (Ser51) phosphorylation (Fig. 6A) and 4E-BP1 protein expression (Fig. 6B) in colonic muscle strips, suggesting an important role of norepinephrine in upregulating the translation of mRNA. Similarly, LiCl treatment of muscle strips markedly attenuated p-eIF2α (Ser51) and 4E-BP1 (data not shown), which suggests that GSK-3β phosphorylation is involved in upregulation of mRNA translation in response to norepinephrine. We also treated muscle strips with Akti-1/2 for 30 min, followed by 6-h incubation with 1 μM norepinephrine. 0.5 μM Akti-1/2 almost completely abrogated the downregulation of 4E-BP1 (Ser51) and 4E-BP1 (data not shown), indicating that Akt mediates norepinephrine-induced translation.

Norepinephrine specifically enhances α1C-subunit mRNA translation. We tested whether norepinephrine augments global translation or α1C mRNA-specific translation. We quantified newly synthesized proteins by measuring [14C]phenylalanine uptake. New protein synthesis increased time dependently, as expected (Fig. 7A). The incubation of muscle strips with norepinephrine or Akti-1/2 plus norepinephrine had no significant effect on [14C]phenylalanine uptake, indicating lack of norepinephrine effect on total new protein synthesis (Fig. 7A). SDS-PAGE analysis of whole cell lysates, followed by visualization with autoradiography, showed no significant difference among control and norepinephrine-treated muscle strips (Fig. 7B, left), further confirming that norepinephrine does not alter global translation. Next, we immunoprecipitated total α1C-protein with anti-α1C antibody, separated precipitated proteins by SDS-PAGE, and visualized newly synthesized α1C by autoradiography. New α1C-protein level was significantly increased by norepinephrine and the increase was abrogated by Akti-1/2 (Fig. 7B, right and Fig. 7C), suggesting that norepinephrine specifically stimulates α1C mRNA translation in colonic muscularis externa and that Akt mediates norepinephrine-enhanced translation of α1C mRNA.

Chronic stress in conscious rats phosphorylates Akt and GSK-3β. We investigated the physiological relevance of in vitro findings by subjecting conscious rats to a 9-day HeICS protocol to investigate whether it activates the PI3K/Akt/ GSK-3β signaling pathway in colonic smooth muscle cells. This stress protocol was reported previously to significantly elevate norepinephrine in rat plasma and α1C-protein in colonic circular smooth muscle cells (8). Immunoblotting of the muscularis externa homogenates collected 8 h after the last stressor of the 9-day HeICS protocol showed significant augmentation of α1C, pAkt, and pGSK-3β (Fig. 8, A–C).

![Fig. 6](http://ajpgi.physiology.org)

**Fig. 6.** NE suppresses eIF2α (Ser51) phosphorylation and 4E-BP1 protein level in colonic muscle strips. Colonic muscularis externa was treated with 1 μM NE or 20 mM LiCl. N = 3 each. *P < 0.05 vs. time 0. A: NE time dependently suppressed p-eIF2α (Ser51). B: NE treatment attenuated 4E-BP1; the suppression recovered by Akti-1/2 treatment. C: Akt inhibitor Akti-1/2 almost completely abrogated the downregulation of p-eIF2α (Ser51) by NE. D: Akti-1/2 blocks the suppression of 4E-BP by NE.

![Fig. 5](http://ajpgi.physiology.org)

**Fig. 5.** A: H89 (PKA inhibitor), calphostin C (PKC inhibitor), and KN-93 (CaMKII antagonist) do not suppress NE-induced α1C-protein accumulation in the rat colonic muscularis externa. The antagonists were added to complete DMEM medium containing muscle strips 30 min prior to 6-h incubation with NE. B: MAP kinases inhibitors SB239063 (p38), SP600125 (JNK), and PD98059 (ERK) were unable to suppress α1C-protein induction by NE. At 30 min after incubation with antagonists, muscle strips were treated with 1 μM NE for 6 h. Data are representative of 3 independent experiments.
DISCUSSION

GSK-3, comprised of two isoforms GSK-3α and GSK-3β with 97% sequence homology in their kinase domains, belongs to a family of highly conserved proline-directed serine/threonine protein kinases (22). GSK-3 is a nodal molecule that receives input from multiple upstream signaling pathways (20) and it activates downstream targets (27a) to regulate diverse functions (10, 15), including oncogenesis, metabolic disorders, Alzheimer’s disease, embryonic development, and cell proliferation. GSK-3 is a negatively regulated kinase. It is active in resting cells and its phosphorylation inactivates it. We found that GSK-3β is the predominant form of GSK-3 in colonic smooth muscle cells (data not shown). Our findings show novel targets of GSK-3β signaling in mediating the maladaptive effects of chronic stress by rapidly upregulating expression of the pore-forming α1C-subunit of Cav1.2b channels in colonic smooth muscle cells. Previous studies have identified growth factors, such as insulin, insulin growth factor-1, Wnt, and hypothyroidism as the inactivators of GSK-3β. We found that norepinephrine release from the adrenal medulla (8) following chronic stress is a robust inactivator of GSK-3β.

GSK-3β is a key component of the canonical Wnt and insulin/growth factor signaling pathways. Insulin/growth factors inactivate GSK-3β by phosphorylation at Ser9 and GSK-3α by phosphorylation at Ser21, which is catalyzed by Akt (9). GSK-3 catalyzes the phosphorylation and inhibition of its substrates, such as glycogen synthase and eIF2α, resulting in the inactivation of glycogen biosynthesis and global protein synthesis, respectively (13). On the other hand, Wnt signaling phosphorylates GSK-3β in a multiprotein complex comprised of axin, adenomatous polyposis coli, and β-catenin, which prevents the polyubiquitination and proteasomal degradation of β-catenin (1). The translocation of β-catenin to the nucleus activates Wnt target genes by binding to the TCF/LEF (T-cell factor/lymphoid enhancer-binding factor-1) family of transcription factors that tether to DNA (4, 30). There is no cross talk between the Wnt and insulin signaling pathways (13).

We found that norepinephrine treatment in colonic smooth muscle cells also accumulates β-catenin. We identified two TCF/LEF binding moieties at −1843/−1826 and −1582/−1566 of Caenacle (α1C gene) promoter. Even though β-catenin accumulation increased in response to norepinephrine, there was no increase in the transcripts of α1C, suggesting that Caenacle is not a β-catenin target gene. In addition, we did not find any global increase in protein expression, suggesting that, at least in 24 h, chronic stress may not induce hypertrophy/hyperplasia in colonic smooth muscle cells. Note also that the increase in plasma level of norepinephrine after the last stressor peak at ~8 h and it returns to baseline by 24 h (8). Chronic psychological stress is not associated with hypertrophy/hyperplasia in gut smooth muscle cells.

The activation of α1-AR induces calcium influx, whereas activation of β1-, β2-, and β3-AR accumulates cAMP in the cytoplasm (2). It is well established that these second messengers activate numerous kinases, including PKA, PKC, MAPKs, and CaMKII (2). All of these kinases have been reported to phosphorylate specific serine/threonine residues on the α1C- and β-subunits to enhance short-term Ca,1.2 channel function (25, 26, 40). However, the inhibition of any of these kinases had no significant effect on the sustained increase in expression of the α1C-subunit protein by norepinephrine. On the other hand, inhibition of the PI3K/Akt/GSK-3β signaling pathway almost completely blocked enhancement of the α1C-subunit.

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Fig. 7. NE upregulates specific proteins in muscle strips. A: colonic muscle strips were incubated with [14C]phenylalanine alone and with NE or NE plus Akti-1/2. NE has no effect on global protein synthesis indicated by [14C]phenylalanine incorporation. B: α1C-protein was immunoprecipitated in the above 3 preparations by using anti-α1C antibody. Precipitated proteins (right) and total proteins (left) were subjected to SDS-PAGE and visualized by autoradiography. C: NE significantly increased α1C mRNA translation (newly synthesized α1C). *P < 0.05. N = 3. No change was observed in global proteins (B, left).

Fig. 8. A 9-day heterotypic intermittent chronic stress (HeICS) protocol was applied to 5 Sprague-Dawley rats; 5 sham-stressed rats served as controls (Ctr). At 8 h after the last stressor, animals were euthanized and colonic muscularis externae of stressed rats. Values represent fold changes. N = 5, *P < 0.05 vs. control.
In a previous report with one concentration of each AR antagonist, we found that \( \alpha_{1C} \) and \( \beta_3 \)-AR regulate the expression of \( \alpha_{1C} \)-subunit protein by norepinephrine (8). A concentration-response curve in this study showed that \( \beta_1/2 \) and \( \beta_3 \)-ARs regulate this expression. We also found significant interr variabili ty of these data with AR antagonists, which may explain the variance. The variability in the data with phentolamine and propranolol might be due to the nonspecific effects of these antagonists at higher concentrations or due to variable expressions of \( \alpha \)- and \( \beta_1/2 \)-ARs in this species. We used \( \beta \)-actin as internal control in each study and expressed the data as \( \alpha_{1C} \)-to-\( \beta \)-actin ratio. The enhancement of \( \alpha_{1C} \)-subunit protein by \( \beta_3 \)-AR agonist confirms the prominent role \( \beta_3 \)-AR in mediating the effects of norepinephrine on the expression of \( \alpha_{1C} \)-subunit.

The influx of calcium through \( \text{Ca}_1.2 \) channels in smooth muscle cells, cardiomyocytes, skeletal muscle, and central nervous system neurons is an essential early step in regulating their cellular functions, including cell excitability and gene expression (14, 17, 18, 24, 28, 32). In muscle cells, the amplitude of the inward calcium current through these channels regulates the amplitude of contractions. Perturbations in calcium current through these channels contribute to the pathogenesis of several diseases, including colonic motor dysfunction in inflammation, hypertrophy, and heart failure (7, 23, 28). These channels are heteromeric transmembrane proteins comprised of the pore-forming \( \alpha_{1C} \)-subunit, auxiliary subunits \( \beta \), disulfide-linked \( \alpha_2 \)-\( \delta \), and sometimes \( \gamma \)-subunits. The pore-forming \( \alpha_{1C} \)-subunit regulates the density of \( \text{Ca}_1.2 \) channels on the cell membrane, whereas the auxiliary \( \beta \)-subunit regulates the gating mechanisms, which determine the amplitude and duration of each episode of calcium current.

The strategic location of these channels at the beginning of signaling cascades that regulate cellular functions in excitable cells makes them an attractive target to modulate organ function in response to psychological and pathogenic stimuli. However, the nature and mechanisms of this modulation are stimulus specific and they depend on the desired modulation of organ function to maintain homeostasis. For example, acute stress resulting in “flight or fight” response requires instantaneous increase in performance of the cardiovascular and skeletal muscle. Rapid release of norepinephrine from the sympathetic neurons followed by swift phosphorylation of the \( \alpha_{1C} \) and \( \beta \)-subunits achieves this response (25, 26, 40). The phosphorylation of these subunits facilitates inward calcium current by increasing its amplitude and duration. This effect is sustainable for short periods and it is reversible by reducing channel subunit phosphorylation by phosphatases (19, 35).

By contrast, the maladaptive response to chronic stress develops over a longer time period and lasts for several hours after the stress is over (8). Previous findings show that chronic stress achieves this goal in colonic smooth muscle cells by enhancing protein expression of \( \alpha_{1C} \)-subunit of the \( \text{Ca}_1.2b \) channels, which increases the density of these channels and, therefore, the amplitude of calcium influx with each depolarization (8, 33). The increase in expression of the \( \alpha_{1C} \)-subunit is mediated also by norepinephrine, but that released from the adrenal gland, rather than from the sympathetic neurons (8). The norepinephrine release from the adrenal gland elevates the plasma level of norepinephrine for several hours, opening up the possibility of altering the expression of target proteins.

The transcriptional upregulation of constitutively expressed proteins, such as \( \alpha_{1C} \)-subunit, is a relatively slow process. The transcriptional upregulation of de novo \( \alpha_{1C} \)-subunit protein in response to vasoactive intestinal polypeptide takes \( \sim 6 \) h (33). However, the increase in \( \alpha_{1C} \)-subunit protein following chronic stress follows closely the increase in plasma norepinephrine (8). Our in vitro findings found steep rates of upregulation and degradation of the \( \alpha_{1C} \)-subunit in response to norepinephrine. In accordance with this, we did not find an increase in transcripts of the \( \alpha_{1C} \)-subunit on incubation of colonic muscularis externa with norepinephrine.

The regulation of translation of transcripts is a complex process. However, our findings show that two critical molecules, 4E-BP1 and p-eIF2\( \alpha \) (Ser51), serve as substrates of GSK-3\( \beta \) to regulate the specific translation of \( \alpha_{1C} \)-mRNA in response to...
norepinephrine release following chronic stress. These molecules are negative regulators of the α1C-subunit translation. Both norepinephrine and LiCl, which enhance the translation of the α1C, suppress the expression of 4E-BPI and p-eIF2α.

In summary, the responses to both, acute and chronic stresses, require quick action to maintain homeostasis. In specific pathways of these two molecules remain to be identified. The inactivation of GSK-3β, 4E-BP1 and the phosphorylation of eIF2α/H9251/H9252 identify novel substrates of GSK-3β. The inactivation of GSK-3β by norepinephrine suppresses the phosphorylation of α1C, resulting in inhibition of its polyubiquitination and proteasomal degradation (Fig. 9). Concurrently, the inactivation of GSK-3β, suppresses the expression of 4E-BPI and the phosphorylation of eIF2α, both of which regulate global and gene specific mRNA translation (12). The specific pathways of these two molecules remain to be identified. Together, the concurrent inhibition of α1C-protein degradation and increase in the translation of its existing transcripts leads to its rapid enhancement in less than 45 min, which is severalfold faster than the rate of transcriptional enhancement of this protein, ~6 h (33).

REFERENCES


