High Glucose Activates Nuclear Factor of Activated T Cells in Native Vascular Smooth Muscle

Jenny Nilsson, Lisa M. Nilsson, Yung-Wu Chen, Jeffery D. Molkentin, David Erlinge, Maria F. Gomez

Objective—Hyperglycemia has been suggested to play a role in the development of vascular disease associated with diabetes. Atypical Ca\(^{2+}\) signaling and gene expression are characteristic of vascular dysfunction; however, little is known regarding the effects of high glucose on Ca\(^{2+}\)-dependent transcription in the vascular wall.

Methods and Results—Using confocal immunofluorescence, we show that modest elevation of extracellular glucose (ie, from 2 to 11.5 mmol/L) increased [Ca\(^{2+}\)], leading to nuclear accumulation of nuclear factor of activated T cells (NFAT) in intact cerebral arteries from mouse. This was accompanied by increased NFAT-dependent transcriptional activity. Both the increase in Ca\(^{2+}\) and NFAT activation were prevented by the ectonucleotidase apyrase, suggesting a mechanism involving the release of extracellular nucleotides. We provide evidence that the potent vasoconstrictors and growth stimulators UTP and UDP mediate glucose-induced NFAT activation via P2Y receptors. NFAT nuclear accumulation was inhibited by the voltage-dependent Ca\(^{2+}\) channel blockers verapamil and nifedipine, the calcineurin inhibitor cyclosporine A, and the novel NFAT blocker A-285222. High glucose also regulated glycogen synthase kinase 3β and c-Jun N-terminal kinase activity, yielding decreased kinase activity and reduced export of NFAT from the nucleus, providing additional mechanisms underlying the glucose-induced NFAT activation.

Conclusions—Our results identify the calcineurin/NFAT signaling pathway as a potential metabolic sensor for the arterial smooth muscle response to high glucose. (Arterioscler Thromb Vasc Biol. 2006;26:0000-0000.)

Key Words: NFAT ■ high glucose ■ vascular smooth muscle ■ extracellular nucleotides ■ GSK-3

Glucose has been shown to activate nuclear factor of activated T cells (NFAT) in pancreatic β-cells, promoting insulin gene transcription. Previous studies have shown that this transcription factor is expressed in native vascular smooth muscle and can be activated in response to vasoconstrictor agonist stimulation. Furthermore, NFAT inhibition has been shown recently to reduce balloon injury–induced neointima formation in rat carotid artery. However, it is unknown whether glucose can activate NFAT in the vasculature.

There are 4 well-characterized members of the NFAT family, all of which depend on dephosphorylation by calcineurin to translocate to the nucleus. In immune cells, activation of this signaling pathway leads to production of cytokines and T-cell proliferation. Inhibition of NFAT nuclear translocation is largely responsible for the immunosuppressive actions of cyclosporine A and tacrolimus (FK506), which specifically block calcineurin. Although originally thought to be restricted to T cells, NFAT has since been shown to regulate heart valve development, skeletal muscle differentiation, and vascular development during embryogenesis.

NFAT has also been implicated in the pathogenesis of cardiac and skeletal muscle hypertrophy and might be predicted to play a role in smooth muscle hypertrophy associated with, for example, hypertension and atherosclerosis. Indeed, Amberg et al recently demonstrated that sustained angiotensin II stimulation, which in vivo results in hypertension, leads to NFAT activation in arterial smooth muscle. This activation causes decreased expression and function of voltage-dependent K\(^{+}\) (Kv) channels, resulting in enhanced vascular excitability.

In arterial smooth muscle, the extracellular nucleotide UTP and its degradation product uridine diphosphate UDP, acting on membrane P2Y\(_{\mathrm{UR}}\) and P2Y\(_{\mathrm{UR}}\) receptors, are potent growth factors stimulating cell cycle progression, cell division, and cellular hypertrophy. UTP raises intracellular Ca\(^{2+}\) and engages multiple signaling pathways, resulting in increased NFATc3 nuclear accumulation, which is the predominant isoform in this tissue. This is dependent on inositol triphosphate receptor-mediated release of Ca\(^{2+}\) from intracellular stores, extracellular Ca\(^{2+}\) influx from voltage-dependent Ca\(^{2+}\) channels (VDCCs), and calcineurin activity.
UTP effectively promotes NFATc3 nuclear accumulation not only by appropriate tuning of the Ca\(^{2+}\) signal but also by suppressing the activity of c-Jun N-terminal kinase (JNK), which phosphorylates and promotes the export of NFATc3 from the nucleus. The subcellular localization of NFATc3 therefore reflects the dynamic interplay between the cytosolic phosphatase activity of calcineurin, promoting NFAT nuclear import, and the activity of this serine-threonine kinase. Among other kinases, evidence obtained in transfection systems suggests that the glycogen synthase kinase 3 (GSK-3) can counteract NFAT nuclear accumulation. Both in cardiac and skeletal muscle, activation of GSK-3 suppresses hypertrophy via inhibition of NFAT signaling.

Apart from promoting NFAT export, GSK-3 inactivates glycogen synthase, the last enzyme in glycogen biosynthesis. Normally, when the glucose availability is high, GSK-3 activity is inhibited and glycogen can be stored. Insulin and growth factors, via phosphatidylinositol 3-kinase, inhibit GSK-3 acutely, leading to increased glycogen and protein synthesis. Interestingly, in vascular smooth muscle, phosphorylating GSK-3 kinase has been suggested to increase NFAT activation in response to very low-density lipoproteins via phosphorylation and thus inactivation of GSK-3. This kinase is also a downstream target of mTor, the substrate of the immunosuppressant rapamycin.

In this study, we test the hypothesis that changes in extracellular glucose levels may activate NFAT in vascular smooth muscle.

**Methods**

**Tissue Samples**

Aortas and cerebral arteries from NMRI mice and aortas and portal veins from NFAT-luciferase transgenic mice were used. Mice were euthanized by cervical dislocation in accordance with approved local ethical guidelines.

**Immunofluorescence**

Experiments were performed as described previously. Primary antibody, rabbit anti-NFATc3 (Santa Cruz Biotechnology), and Cy5-labeled secondary antibody (Jackson ImmunoResearch Laboratories) were used. Nuclei were stained with the nucleic acid dye SYTOX Green (Molecular Probes). NFATc3 and nuclear regions were detected by monitoring Cy5 and green fluorescence on a Zeiss LSM 510 laser scanning confocal microscope. For scoring of NFATc3-positive nuclei, 3 to 5 fields for each vessel were imaged, and an average of 277 cells per field was counted under blind conditions. A cell was considered positive if colocalization (white) was observed in the nucleus and negative if no colocalization (green only) was visualized.

**Luciferase Reporter Assay**

Luciferase enzymatic activity in arteries from phenotypically normal NFAT-luciferase transgenic mice was determined using a commercial kit (Promega) according to the supplier indications. Optical density was measured (VICTOR 3 multilabel counter; Perkin-Elmer) and expressed as relative luciferase units normalized to protein concentration.

**Confocal Ca\(^{2+}\) Measurements**

Experiments were performed as described previously. Arteries were loaded with fluo-4-AM and imaged using a Zeiss LSM 510 laser-scanning confocal microscope. Images were acquired every 1.56 s, before and after each treatment, and changes in global fluorescence (\(F/F_0\)) were calculated.

**Western Blot Analysis**

The following antibodies were used: goat polyclonal anti-GSK-3β, anti-p-GSK-3β (Santa Cruz Biotechnology), and rabbit polyclonal anti-JNK1&2 (BioSource International, Inc.) on cerebral arteries and aortas.

**Chemicals**

Fluo-4 and pluronic acid were from Molecular Probes, Inc., GSK-3β inhibitor was from Calbiochem, MRS2578 was a gift from Dr K.A. Jacobson (National Institutes of Health; Bethesda, Md), and A-285222 was provided by Abbott Laboratories. All other drugs were from Sigma.

**Statistical Analysis**

Results are expressed as means±SEM. Statistical significance was determined using 1-way ANOVA followed by Bonferroni or Tukey-Kramer tests (for comparisons between <5 and >6 groups, respectively).

For a detailed version of the methods, please see the online supplement, available at http://atvb.ahajournals.org.

**Results**

**Glucose Acutely Induces NFATc3 Activation**

In intact cerebral arteries, raising the extracellular glucose concentration from 11.5 mmol/L (control) to 20 mmol/L (high glucose [HG]) for 30 minutes significantly increases NFATc3 nuclear accumulation (Figure 1A). The number of NFATc3-positive nuclei increases from 19% in control vessels to 49% in arteries exposed to HG (Figure 1B). This was prevented by the calcineurin inhibitor cyclosporin A (1 μmol/L) and by the novel NFAT blocker A-285222 (1 μmol/L; Figure 1B). Mannitol had no effect on NFATc3 nuclear accumulation, ruling out a possible osmotic effect of glucose; and L-glucose, which cannot be metabolized by the cell, had no effect either (Figure 1B).

Exposure to HG for 30 minutes also resulted in increased NFAT-dependent luciferase activity in aorta and portal vein from mouse (Figure 1C), indicating that nuclear accumulation of NFAT is accompanied by enhanced transcriptional activity. Corresponding experiments on cerebral arteries were hampered by low tissue yield.

**Time and Dose Dependency of Glucose-Induced NFAT Activation**

Time- and dose-response experiments reveal significantly increased NFATc3 nuclear accumulation after 8-minute exposure to HG and a stepwise response when raising the extracellular glucose concentration >11.5 mmol/L (Figure 2A and 2B). A 3.5-mmol/L increase (to 15 mmol/L) was sufficient to achieve significant NFATc3 nuclear accumulation, whereas lowering glucose to 8 or 5 mmol/L had no effect (Figure 2B).

For this study, 11.5 mmol/L was considered as control glucose concentration because all our previous NFAT data in cerebral arteries were obtained using PSS containing this level of glucose. This level is higher than the levels the arteries may face in vivo, but it constitutes the only energy source during in vitro experiments and was therefore chosen as “control” condition. Because the lack of response at lower
glucose concentrations (5 and 8 mmol/L) could be attributed
to the elevated basal level used as control, we used a different
experimental paradigm in which cerebral vessels were al-
lowed to equilibrate in media containing 2 mmol/L glucose
overnight and were then stimulated for 30 minutes with
11.5 mmol/L or 20 mmol/L glucose. This resulted in a
dose-dependent and significant increase in NFATc3 nuclear
accumulation (Figure 3A). Similar experiments using aortas
from NFAT-luciferase mice show that stimulation with media
containing 7, 11.5, or 20 mmol/L glucose after overnight
equilibration in 2 mmol/L glucose yields enhanced NFAT-
dependent transcriptional activity (Figure 3B). Interestingly,
the level of NFATc3 nuclear accumulation or NFAT-
dependent transcriptional activity after overnight incubation
with 2 mmol/L was higher than the levels measured in
noncultured arteries, suggesting that culture per se may result
in NFAT activation (Figure 1A and 1B versus 1C).

**Release of Extracellular Nucleotides Mediates**
**Glucose-Induced NFATc3 Nuclear Accumulation**
The ectonucleotidase apyrase (0.32 U/mL) prevents HG-
induced NFATc3 nuclear accumulation (Figure 4A) and
NFAT-dependent luciferase activity (Figure 1C), suggesting
enhanced release of extracellular nucleotides on HG expos-
ure. MRS2578, a selective antagonist of the UDP receptor
P2Y<sub>6</sub> significantly decreased glucose-induced NFATc3 nu-
clear accumulation (Figure 4A), providing further evidence
for the involvement of extracellular nucleotides in this
response.

UTP was slightly more effective than the stable pyrimi-
dines UDP<sub>B</sub> (selective for P2Y<sub>6</sub> receptors) and UDP<sub>S</sub>
(selective for P2Y<sub>2/4</sub> receptors), in stimulating NFATc3 nu-
clear accumulation (Figure 4B). It is possible that the less
stable UTP may act both on P2Y<sub>2/4</sub> receptors and, after
degradation to UDP, on P2Y<sub>6</sub> receptors. As shown in Figure
1C, UTP also effectively increased NFAT-dependent lucif-
erase activity.

**Figure 1.** HG activates NFAT in vascular smooth muscle. A, Representative images showing cytosolic localization of NFATc3 in control
condition (11.5 mmol/L glucose) and nuclear localization on 30-minute HG (20 mmol/L) in cerebral arteries, with or without the NFAT
inhibitor A-285222 (1 μmol/L). White indicates nuclear colocalization of NFATc3 (red) and the DNA-binding dye SYTOX (green).

Bars = 20 μm. B, Percentage of cells exhibiting NFATc3 nuclear accumulation in cerebral vessels treated with HG for 30 minutes with or
without cyclosporin A (1 μmol/L; cyclosporine A [CsA]) or A-285222 (1 μmol/L), mannitol, and L-glucose (both 20 mmol/L; 30 minutes).
Experiments were performed at room temperature. ***P<0.001, HG vs all other bars (m indicates No. of mice; v, No. of vessels). C, NFAT-dependent transcriptional activity in aortas and portal veins from transgenic mice. Vessels were exposed to HG with and without
apyrase (0.32 U/mL) or to UTP (100 μmol/L) for 30 minutes and collected 6 hours later. Data expressed as optical density normalized
to protein concentration (relative luciferase units/μg protein). Values from nontransgenic FVBN mice (WT) are shown as negative con-
trols. Experiments are performed in triplicate (m=8; ***P<0.001, **P<0.01, *P<0.05).

**Figure 2.** Time and dose response of glucose-induced NFATc3 nuclear accumulation. A, After treatment with HG for 3, 5, 8, 10,
20, and 30 minutes. B, In response to varying doses of glucose (5, 8, 11.5, 13, 14, 15, 20, and 25 mmol/L) for 30 minutes.
***P<0.001, **P<0.01, *P<0.05, vs control (m indicates No. of mice; v, No. of vessels).

**Figure 3.** Dose response after overnight equilibration in low-glucose media. A, Summarized NFATc3 nuclear accumulation in
arteries equilibrated overnight in 2 mmol/L glucose media and subsequently stimulated for 30 minutes with 11.5 or 20 mmol/L
glucose. B, NFAT-dependent transcriptional activity in aortas equilibrated overnight as in A and then incubated for 6 hours in
media containing 7, 11.5, or 20 mmol/L glucose. Data expressed as optical density normalized to protein concentration
(relative luciferase units/μg protein). ***P<0.001, **P<0.01,
*P<0.05, vs 2 mmol/L (m indicates No. of mice; v, No. of vessels).
Hydrolysis of extracellular nucleotides by apyrase can lead to increased levels of adenosine and, consequently, to activation of K<sub>ATP</sub> channels, hyperpolarization of the cell membrane, and reduced influx of Ca<sup>2+</sup> via VDCCs. The K<sub>ATP</sub> channel opener pinacidil has been shown previously to inhibit NFATc3 nuclear accumulation in these arteries. Hence, adenosine could be predicted to decrease glucose-induced NFATc3 nuclear accumulation by virtue of its effects on K<sub>ATP</sub> channels. To test this, we tried apyrase in the presence of the adenosine inhibitor 2-theophylline (10 μmol/L). Blockade of adenosine failed to affect the inhibition of glucose-induced NFATc3 nuclear accumulation achieved by apyrase, excluding an indirect effect of apyrase other than reduction of available extracellular nucleotides (Figure 4).

In arterial smooth muscle, extracellular nucleotides (ie, ATP, ADP, UTP, and UDP) increase global [Ca<sup>2+</sup>]. Here we show that HG increases global [Ca<sup>2+</sup>], in intact cerebral arteries (Figure 5A through 5C). If activation of P2Y receptors by a glucose-stimulated increase in extracellular nucleotides is responsible for the observed increase in [Ca<sup>2+</sup>], then treatment with apyrase would be expected to prevent a raise in [Ca<sup>2+</sup>]. Indeed, no increase of Ca<sup>2+</sup> was observed when cerebral arteries were exposed to HG in the presence of apyrase (Figure 5C). The L-type VDCC blockers verapamil (10 μmol/L) and nifedipine (100 nmol/L) partially inhibited HG-induced NFATc3 nuclear accumulation (supplemental Figure I, available online at http://atvb.ahajournals.org), indicating that Ca<sup>2+</sup> influx via these channels participates in this response.

GSK-3β Negatively Regulates NFATc3 Nuclear Accumulation

Intact cerebral arteries treated for 30 minutes with the cell-permeable GSK-3β inhibitor Myr-N-GKEAPPAPPQsp-P–NH<sub>2</sub> induced a robust NFATc3 nuclear accumulation, resulting in levels comparable to those observed after HG exposure (Figure 6A). This indicates that GSK-3β is tonically active and contributes to NFATc3 export regulation in these arteries. Also, simultaneous incubation of vessels with the GSK-3β inhibitor and HG failed to achieve higher levels of nuclear NFATc3 than those observed after HG alone, consistent with the effects of glucose on NFATc3 activation being, at least in part, mediated by inhibition of GSK-3β export activity.

Glucose Downregulates GSK-3β and JNK Activity

Raising the glucose concentration from 11.5 to 20 mmol/L for 30 minutes increased GSK-3β phosphorylation by 66% in intact cerebral arteries (Figure 6B and 6C). Insulin (100 nmol/L), which has proven to effectively increase GSK-3β phosphorylation in human skeletal muscle, yielded increased GSK-3β phosphorylation by 55%. We have previ...
tools for dissecting the NFAT-signaling pathway. Instead, the HG-induced NFATc3 activation in these arteries.14 HG-This response is consistent with previous data reported for 

$GSK-3\beta$ phosphorylation on 30-minute HG stimulation. C, Summarized levels of phosphorylated GSK-3\(\beta\) (P-GSK-\(\beta\)), normalized to total GSK-3\(\beta\), expressed as percentage of control (3 separate experiments including cerebral arteries from 14 mice). The effect of insulin (100 nmol/L) is shown for comparison (value from a single experiment with pooled arteries from 10 mice).

**Discussion**

This study shows that changes in the extracellular glucose concentration are readily detected by NFATc3 in native cerebral artery smooth muscle, leading to increased NFATc3 nuclear accumulation and NFAT-dependent transcriptional activity. We therefore propose a role for NFAT as a metabolic sensor in the vascular wall of potential relevance for vascular dysfunction in diabetes. The effect of glucose on NFATc3 activity involves the release of extracellular nucleotides acting on P2Y receptors, leading to increased [Ca\(^{2+}\)], and subsequent activation of calcineurin and NFATc3. Normally, the subcellular localization and transcriptional activity of NFAT reflects a dynamic balance between stimuli promoting nuclear translocation but also decreases the export of NFAT from the nucleus by inhibiting the otherwise constitutively elevated kinase activity of GSK-3\(\beta\) and JNK.

HG-induced nuclear accumulation of NFATc3 is robust and comparable to activation levels obtained after agonist stimulation with UTP or endothelin-1. The time course of this response is consistent with previous data reported for UTP-induced NFATc3 activation in these arteries.14 HG-induced NFATc3 nuclear accumulation is prevented by A-285222, which is a 3,5-bis(trifluoromethyl)pyrazole derivative recently identified as NFAT blocker in immune cells.20,21,26 Traditionally, the NFAT pathway has been studied using calcineurin blockers, such as cyclosporin A, and FK506. Because calcineurin not only interacts with NFAT but also with other substrates (ie, the type II regulatory subunit of protein kinase A,\(^{21}\)), these drugs are ambiguous for dissecting the NFAT-signaling pathway. Instead, the A-285222 compound has been shown to maintain NFAT in a phosphorylated state by a mechanism independent of calcineurin activity.

Regarding the mechanism of action underlying HG-induced NFAT activation, a role for extracellular nucleotides is strongly suggested because the response was blocked by apyrase. This is consistent with studies in endothelial and pancreatic \(\beta\)-cells showing that extracellular nucleotides such as UTP and ATP are released on mechanical stress and increased extracellular glucose concentration.27–29 In the vasculature, autocrine or paracrine release of nucleotides can lead to both vasoconstriction and growth stimulation depending on the activation of specific nucleotide receptor subtype.24 In human and rat cerebral arteries, the UDP receptor P2Y\(_6\), has been shown to play a prominent role in the regulation of vascular tone.30,31 The observation that the P2Y\(_6\) receptor antagonist MRS2578 partially decreased HG-induced NFAT nuclear accumulation provides further evidence for nucleotide release on HG stimulation and highlights the possible engagement of multiple purinergic receptors. A detailed characterization of the nature of the nucleotides released and receptors involved is beyond the scope of this study, but the experiments with the stable analogs UDP\(\beta\)s and UTP\(\gamma\)s, suggest that UTP acting on P2Y\(_{\gamma}\) receptors and UDP acting on P2Y\(_{\delta}\) receptors are strong candidates.

Consistent with previous data,\(^{32}\) an increase in global [Ca\(^{2+}\)], was observed on exposure to HG in cerebral artery smooth muscle. This was prevented by apyrase, suggesting that the increase in Ca\(^{2+}\) is attributable to autocrine/paracrine activation of P2Y purinergic receptors by released extracellular nucleotides. It was shown previously that UTP-induced NFATc3 nuclear accumulation requires both the release of Ca\(^{2+}\) from intracellular stores and influx of Ca\(^{2+}\) via VDCCs because inhibition of either pathway completely abrogates the response.2 Interestingly, HG-induced NFATc3 activation was only partially reduced by inhibition of VDCCs, as shown by experiments using verapamil and nifedipine, highlighting potential differences in the Ca\(^{2+}\) signaling pathways engaged in response to glucose with those of UTP.
UTP not only acts through multiple mechanisms to increase \([Ca^{2+}]\), but also by suppressing relatively elevated basal JNK2 activity.\(^{14}\) A model describing a dual signal mechanism for induction of NFATc3 in arterial smooth muscle was proposed, requiring both a calcineurin-activating \(Ca^{2+}\) signal and engagement of pathways that downregulate NFAT nuclear export. The data presented here are supportive of this model and describe for the first time in native arterial smooth muscle the involvement of another kinase in NFATc3 export regulation: GSK-3β. This kinase is constitutively active in this tissue because pharmacological inhibition of GSK-3β resulted in enhanced NFATc3 nuclear accumulation. The fact that HG also decreases the levels of phosphorylated JNK further supports the concept of HG acting through nucleotides, even if a direct effect of HG on JNK cannot be ruled out.

The role of NFAT in vascular smooth muscle is still unclear; however, NFATc3 may be predicted to face considerable levels of \([Ca^{2+}]\), and calcineurin activation under pressurized conditions. Indeed, recent work by Gonzalez Bosc et al demonstrates that acute increases in intraluminal pressure stimulate NFATc3 nuclear accumulation in mouse cerebral arteries.\(^{33}\) Thus, the presence of \(\approx2\) constitutively active kinases able to suppress NFAT activity provides additional levels of regulation of potential interest in response to metabolic changes or agonist stimulation. The mechanism connecting increases in glucose to the inactivation of GSK-3β is currently unknown, but enhanced GSK-3β phosphorylation in vascular smooth muscle cells (VSMCs) during neointima formation has been reported as a consequence of upregulated glucose transport and metabolism.\(^{34}\)

The described link between HG levels and NFAT activation mediated by activation of P2Y receptors by released extracellular nucleotides is interesting from the clinical perspective. Diabetic patients experience microvascular disease characterized by increased wall-lumen ratio mainly because of increased amounts of vascular smooth muscle cells. Our findings, combined with the potent growth stimulatory effects of both UTP and UDP,\(^{12,13}\) could represent a new mechanism contributing to diabetic microvascular disease. The described NFAT-dependent regulation of Kv channels, leading to enhanced excitability of arterial smooth muscle,\(^{11}\) may represent a contributing factor to vascular dysfunction in the diabetic situation.

Diabetic patients have higher rates of restenosis after coronary angioplasty, resulting in increased morbidity and mortality. The mechanism of restenosis is excessive growth of VSMCs, creating a neointima. UTP has been shown to contribute to neointimal development.\(^{35}\) In diabetic patients, drug-eluting stents coated with sirolimus (rapamycin) or paclitaxel have been valuable to avoid restenosis.\(^{36}\) Interestingly, sirolimus is closely related to the NFAT inhibitors tacrolimus and cyclosporine and reduces GSK-3 activity via inhibition of mTor (see Introduction). It is possible that our results provide a link between diabetes and increased restenosis rates, involving HG-induced release of extracellular nucleotides acting on P2Y receptors and concomitant GSK-3β inhibition to stimulate VSMC growth via NFAT activation. Although a basal glucose level of 11.5 is typical for in vitro experiments, it would be considered hyperglycemic in vivo. Nevertheless, the narrow concentration range of the response (from 11.5 to 15 mmol/L) is interesting because plasma glucose concentrations >11.1 mmol/L are considered in clinical practice as indicative for diabetes.\(^{37}\) The experiments using 2 mmol/L glucose as basal level provide further evidence for a glucose-responsive NFAT pathway at physiologically relevant hyperglycemic levels. The vessels are able to adjust to the glucose environment they are exposed to (11.5 or 2 mmol/L) by equilibrating at relatively low levels of NFAT nuclear accumulation, allowing the system to sense further changes in extracellular glucose and responding with increased nuclear accumulation and transcriptional activity. This may be important in the clinical situation because modest fluctuations (of a few mmol/L) in plasma glucose or hyperglycemic peaks may be sufficient for NFAT activation. In conclusion, modest elevations in extracellular glucose lead to increased NFATc3 nuclear accumulation and NFAT-dependent transcriptional activity in arterial smooth muscle. We therefore propose a role for NFAT as a metabolic sensor in the vascular wall of potential relevance for vascular dysfunction in diabetes. The effect of glucose on NFATc3 nuclear activation involves the release of extracellular nucleotides acting on P2Y receptors, leading to increased intracellular \(Ca^{2+}\) levels and subsequent activation of calcineurin, combined with inhibition of GSK-3β and JNK, leading to reduced nuclear export of NFATc3 (for diagram of proposed mechanisms, please see the online supplement).

**Acknowledgments**

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**References**

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Detailed methods

Tissue Samples — Adult female NMRI mice (20-25 g) were euthanized by cervical dislocation in accordance with approved local ethical guidelines. Aortas and cerebral arteries (anterior, midcerebral, posterior, cerebellar, and basilar) were dissected out in ice-cold physiological saline solution (PSS, containing, in mmol/liter: NaCl, 135; KCl, 5.9; MgCl₂, 1.2; Hepes, 11.6; glucose, 11.5; pH 7.4). For overnight experiments, vessels were cultured in DMEM and Ham’s F12 (1:1) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin, in a water-jacketed incubator (5% CO₂, 37 °C).

Immunofluorescence — All experiments were performed as previously described. Arteries were treated as specified in the text, and mounted onto glass slides. Primary antibody, rabbit anti-NFATc3 (Santa Cruz Biotechnology; 1:250) was applied overnight at 4 °C. Secondary antibody, Cy5-anti-rabbit IgG (Jackson ImmunoResearch Laboratories; 1:500), was applied for 1 h at RT. Nuclei were stained with the fluorescent nucleic acid dye SYTOX Green (Molecular Probes, 1:3000). NFATc3 and nuclear regions were detected by monitoring Cy5 (excitation 633, emission > 650 nm) and green fluorescence (excitation 488, emission 505-530 nm), at 40X on a Zeiss LSM 510 laser scanning confocal microscope. The crosshair function of the colocalization tool of the LSM 510 system (Zeiss) was used for visualization of colocalized image regions, leading to the distribution of all image pixels over 4 quadrants in a scattergram according to their intensity levels: Background pixels sorted into the bottom left quadrant, single-tagged pixels (either red or green) into the upper left and bottom right quadrants, and pixels having an intensity above the background in both channels (i.e. colocalized pixels, color-
coded white in the original image) represented by the upper right quadrant. For scoring of NFATc3-positive nuclei, 3-5 fields for each vessel were imaged and an average of 277 cells per field was counted under blind conditions (the number of vessels, \( v \), and mice, \( m \), is indicated in each figure). A cell was considered positive if co-localization (white) was observed in the nucleus; whereas a cell was considered negative if no co-localization (green only) was visualized. Levels of NFATc3 nuclear accumulation are expressed as percentage of NFATc3 nuclear accumulation (number of positive nuclei relative to total number of nuclei).

*Luciferase Reporter Assay*—Phenotypically normal, female NFAT-luc transgenic mice were used \(^3\). These mice express nine copies of an NFAT binding site from the interleukin-4 promoter, positioned 5' to a minimal promoter from the \( \alpha \)-myosin heavy chain gene (-164 to +16) and inserted upstream of a luciferase reporter gene. Aortas and portal veins were treated as specified in the text and homogenized in lysis buffer: 100 mM KPO\(_4\) (pH 7.8), 0.5% NP-40 and 1 mM DTT. An aliquot of supernatant, obtained by centrifugation at 13200 \( \times g \) for 15 min, was added to luciferase substrate reagent: 100 mM Tris-HCl (pH 7.8), 10 mM MgAcetate, 1 mM EDTA, 1.4 mM luciferin (Promega) and 18.3 mM ATP. Optical density was measured (VICTOR 3 multilabel counter, PerkinElmer), normalized to protein concentration as determined by Bradford assay (BioRad) and expressed as relative luciferase units.

*Confocal Ca\(^{2+}\) Measurements* - All imaging experiments were performed at RT as previously described \(^2\). Arteries loaded with 10 \( \mu \)M fluo-4-AM in PSS and 0.05%
pluronic acid for 30 min were illuminated with a krypton/argon laser at 488 nm and imaged using a Zeiss LSM 510 laser-scanning confocal microscope (40X oil immersion lens, numerical aperture = 1.3). Images of the vessel wall were acquired every 1.56 s (0.69 images/s), before and after the corresponding treatment. Changes in global fluorescence ($F/F_0$) were calculated by measuring the fluorescence intensity ($F$) within 5-10 boxes (3.2 μm X 3.2 μm, or 10 X 10 pixels) randomly placed within each artery, divided by a baseline ($F_0$) that was determined by averaging fluorescence intensity 20 s before application of the drug.

**Western Blot Analysis** – Cerebral arteries and aortas were homogenized in lysis buffer. Proteins were separated by SDS-PAGE on 12.5% gels, transferred and blocked for 1 h at RT (Tris-buffered saline with 0.05% Tween 20 and 5% BSA). Blots were incubated with primary antibodies overnight at 4 °C (goat polyclonal anti-GSK-3β, 1:200 and anti-p-GSK-3β; 1:200, both from Santa Cruz Biotechnology; and rabbit polyclonal anti-JNK1&2[pTpY183/185] phosphospecific antibody, 1:1000 from BioSource International, Inc.) washed, and treated with horseradish peroxidase-conjugated secondary antibody for 1 h. Blots were developed using an enhanced chemiluminescence substrate (SuperSignal West Femto or West Pico; Pierce, for GSK-3β and p-JNK respectively). For quantification, gel analysis software was used (Quantity-One, BioRad).

**Chemicals**—All drugs and chemicals were purchased from Sigma unless otherwise specified. Fluo-4 and pluronic acid were from Molecular Probes, Inc. (Eugene, OR). The
GSK3β-inhibitor was from Calbiochem. MRS2578 was a gift from Dr. K. A. Jacobson (NIH, Maryland, USA). A-285222 was kindly provided by Abbott Laboratories, IL.

Statistical Analysis—Results are expressed as means ± S.E.M., where applicable. Statistical significance was determined using one-way analysis of variance followed by Bonferroni or Tukey-Kramer tests (for comparisons between <5 and >6 groups, respectively).

References:
Fig. 1

% NFATc3 nuclear accumulation

Control
HG
HG + Vera
HG + Nifedipine

v=13
m=6

v=12
m=8

v=4
m=3

v=6
m=5

0 25 50 75
Fig. II

A

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B

**P-JNK2**

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**P-JNK1**

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* *
Fig. III
Figure legends

**Fig. I.** Dependence of glucose-induced NFATc3 nuclear accumulation on Ca\(^{2+}\). Partial abrogation of glucose-induced NFATc3 nuclear accumulation with the VDCC-blockers verapamil (10 μM) or nifedipine (100 nM). Asterisks above individual bars indicate differences compared to control, other asterisks represent comparisons as indicated (m=number of mice; v, number of vessels).

**Fig. II.** HG decreases JNK activity in arterial smooth muscle. *A*, western blot showing decreased levels of phosphorylated JNK (P-JNK) upon 30 min UTP (100 μM) or HG stimulation in mouse aorta. Bands corresponding to JNK2 and JNK1 isoforms are shown (~54 and 46 KDa, respectively). *B*, results from 3 separate experiments as in A expressed as % of control, each including arteries from 3-4 mice. Data normalized to total protein loading for each lane, as determined by Coomassie blue staining.

**Fig. III.** Cartoon of proposed mechanisms underlying the effects of HG on NFAT activation. Exposure to HG leads to activation of NFAT in vascular smooth muscle via at least two mechanisms: 1) Release of extracellular nucleotides acting on P2Y receptors, leading to increased [Ca\(^{2+}\)], and activation of calcineurin and 2) inhibition of GSK-3β and JNK, resulting in decreased kinase activity and hence, down-regulation of NFAT nuclear export activity.