Hyperglycemic Conditions Prime Cells for RIP1-dependent Necroptosis*

Received for publication, January 15, 2016, and in revised form, April 25, 2016 Published, JBC Papers in Press, April 29, 2016, DOI 10.1074/jbc.M116.716027

Timothy J. LaRocca†, Sergey A. Sosunov‡, Nicole L. Shakerley‡, Vadim S. Ten‡, and Adam J. Ratner†∥¶

From the †Department of Basic and Social Sciences, Albany College of Pharmacy and Health Sciences, Albany, New York 12208, the ‡Department of Pediatrics, Columbia University, New York, New York 10032, and the Departments of ‡Pediatrics and ¶Microbiology, New York University, New York, New York 10016

Necroptosis is a RIP1-dependent programmed cell death (PCD) pathway that is distinct from apoptosis. Downstream effector pathways of necroptosis include formation of advanced glycation end products (AGEs) and reactive oxygen species (ROS), both of which depend on glycosylation. This suggests that increased cellular glucose may prime necroptosis. Here we show that exposure to hyperglycemic levels of glucose enhances necroptosis in primary red blood cells (RBCs), Jurkat T cells, and U937 monocytes. Pharmacologic or siRNA inhibition of RIP1 prevented the enhanced death, confirming it as RIP1-dependent necroptosis. Hyperglycemic enhancement of necroptosis depends upon glycosylation with AGEs and ROS playing a role. Total levels of RIP1, RIP3, and mixed lineage kinase domain-like (MLKL) proteins were increased following treatment with high levels of glucose in Jurkat and U937 cells and was not due to transcriptional regulation. The observed increase in RIP1, RIP3, and MLKL protein levels suggests a potential positive feedback mechanism in nucleated cell types. Enhanced PCD due to hyperglycemia was specific to necroptosis as extrinsic apoptosis was inhibited by exposure to high levels of glucose. Hyperglycemia resulted in increased infarct size in a mouse model of brain hypoxia-ischemia injury. The increased infarct size was prevented by treatment with nec-1s, strongly suggesting that increased necroptosis accounts for exacerbation of this injury in conditions of hyperglycemia. This work reveals that hyperglycemia represents a condition in which cells are extraordinarily susceptible to necroptosis, that local glucose levels alter the balance of PCD pathways, and that clinically relevant outcomes may depend on glucose-mediated effects on PCD.

Necroptosis is an inflammatory programmed cell death (PCD)2 distinct from apoptosis (1, 2). Necroptosis drives ischemia-reperfusion injury of the brain, kidneys, and heart such that pharmacological inhibition of this PCD reduces infarct size in models of these injuries (3, 4). This PCD is independent of apoptosis and caspases (1, 2). Well studied necroptotic stimuli are ligands of the TNF family, including TNF-α, Fas ligand (FasL), and TNF-like apoptosis-inducing ligand (TRAIL), in the context of pan-caspase inhibition with Z-VAD-fmk (1, 2, 4). Necroptosis was recently shown to occur in anucleate red blood cells (RBCs) in response to the human CD59 (hCD59)-specific bacterial pore-forming toxins (PFTs), vaginolysin (VLY) and intermedilysin (ILY) (5, 6). This PCD depends on the phosphorylation of RIP1 and RIP3 kinases (1, 5, 6). The interaction of RIP1 with RIP3 is necessary for most forms of necroptosis, leading to the cytosolic necrosome complex, which also includes Fas-associated death domain (FADD), caspase-8, and mixed lineage kinase domain-like (MLKL) protein (1, 2). Within this complex, RIP1, RIP3, and MLKL drive downstream effector signaling in necroptosis resulting in cell death (2).

Effector pathways downstream of the necrosome that are responsible for cell death include formation of advanced glycation end products (AGEs) and reactive oxygen species (ROS) (1, 7). Formation of AGEs and ROS depend on glycolysis (8), which is increased during necroptosis (1). AGEs are formed due to the synthesis of the toxic derivative, methylglyoxal, from the fragmentation of glyceraldehyde phosphate and dihydroxyacetone phosphate during glycolysis (8, 9). The production of ROS is predominantly due to premature release of partially reduced oxygen from the electron transport chain of oxidative phosphorylation (10). Another major source of ROS is the NADPH oxidase family of proteins, which are of wide tissue distribution (11). As oxidative phosphorylation and NADPH formation depend on glucose metabolism (10), glycolysis is viewed as a regulator of ROS production.

Hyperglycemia and diabetes lead to an increase in the cellular uptake of glucose (12). As stimulation of glycolysis is a major effector of necroptosis (1, 7), this suggests that this PCD may be enhanced by hyperglycemia. Here we show that exposure to hyperglycemic levels of glucose predispose primary RBCs, Jurkat T cells, and U937 monocytes for RIP1-dependent necroptosis resulting in a significant enhancement of this PCD in vitro. RIP1 was shown to be critical for this through pharmacological inhibition and RNAi. Glycolysis and AGEs were important for this enhancement of necroptosis in all cell types, whereas ROS,

---

* This work was supported by startup funds from the Albany College of Pharmacy and Health Sciences (to T. J. L.) and National Institutes of Health Grants R01-AI092743 and R21-AI111020 (to A. J. R.), and R01-N5088197 (to V. S. T.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors declare that they have no conflicts of interest with the contents of this article.

† To whom correspondence should be addressed: Depts. of Pediatrics and Microbiology, Division of Pediatric Infectious Diseases, New York University School of Medicine, 550 First Ave., MSB 223, New York, NY 10016. Tel.: 646-501-0044; E-mail: Adam.Ratner@nyumc.org.

‡ The abbreviations used are: PCD, programmed cell death; Z, benzylxycarbonyl; fmk, fluoromethyl ketone; PFT, pore-forming toxin; VLY, vaginolysin; ILY, intermedilysin; MLKL, mixed lineage kinase domain-like; AGE, advanced glycation end product; ROS, reactive oxygen species; HI, hypoxia-ischemia; aSMase, acid sphingomyelinase; DMSO, dimethyl sulfoxide.
and increased protein levels of RIP1, RIP3, and MLKL were involved in the enhancement of necroptosis in nucleated cells. Hyperglycemic priming of PCD was specific to necroptosis, as neither apoptosis nor extrinsic apoptosis was enhanced. Brain infarcts, induced by hypoxia-ischemia (HI) injury, were significantly greater size in the setting of hyperglycemia in mice. Exacerbation of brain infarcts was completely prevented by RIP1 inhibition in vivo. These results demonstrate an unprecedented biochemical link between hyperglycemia, glycolysis, and enhanced RIP1-dependent necroptosis.

Experimental Procedures

Cells and Bacterial Strains—Primary human RBCs were obtained from healthy volunteers under a protocol approved by the Columbia University Institutional Review Board. U937 monocytes and Jurkat T cells were cultured in RPMI with 10% fetal bovine serum at 37 °C, 5% CO2.

Hemolysis Assays—Recombinant PFTs were purified as described previously (5, 6, 33). Hemolysis assays were performed in Dulbecco’s PBS at 37 °C for 30 min using 0.1 hemolytic units of VLY or ILY. For this, 1 hemolytic unit is defined as the amount of PFT necessary to induce 50% hemolysis of RBCs exposed to 5 mM glucose, as described previously (5). RBCs were preincubated in Dulbecco’s PBS containing the indicated amounts of glucose for 24 h at 37 °C to induce uptake. RBCs were washed with Dulbecco’s PBS lacking glucose prior to experiments. Inhibitors were incubated with RBCs for 1 h at 37 °C prior to hemolysis assays and used at these concentrations: nec-1s (EMD Millipore, 50 μM), pyridoxamine (Acros Organics, 0.5 mM), desipramine (Tocris, 20 μM), and 2,2-bipyridyl (Alfa Aesar, 50 μM). Hyperosmotic and calcium-induced eryptosis were induced by incubation of RBCs with 950 mM sucrose or 1 mM CaCl2, respectively, for 24 h at 37 °C. Hemolysis was measured by hemoglobin release in a spectrophotometer at 415 nm.

Cell Death Assays—Recombinant human TNF-α (Life Technologies) and FasL (Enzo Life Sciences) were used at the indicated concentrations: nec-1s (EMD Millipore, 25 μM), pyridoxamine (Acros Organics, 0.5 mM), desipramine (Tocris, 20 μM), and 2,2-bipyridyl (Alfa Aesar, 50 μM). Hyperosmotic and calcium-induced eryptosis were induced by incubation of RBCs with 950 mM sucrose or 1 mM CaCl2, respectively, for 24 h at 37 °C. Hemolysis was measured by hemoglobin release in a spectrophotometer at 415 nm.

Cell Death Assays—Recombinant human TNF-α (Life Technologies) and FasL (Enzo Life Sciences) were used at the indicated LD50 doses in the presence of 0.5 μg/ml of cycloheximide. Cell death assays were performed in RPMI at 37 °C, 5% CO2, for 24 h with cells at 5 × 106 cells/ml. Cells were preincubated in RPMI containing the indicated amounts of glucose for 24 h to induce uptake. Cells were washed with normal RPMI prior to experiments. For experiments that utilized sodium pyruvate, 5 mM was added to the cells and allowed to incubate for 24 h. Inhibitors were incubated with cells for 1 h prior to death assays and used at these concentrations: nec-1s (EMD Millipore, 25 μM), Z-VAD-fmk (Takara, 10 μM), pyridoxamine (Acros Organics, 0.5 mM), desipramine (Tocris, 20 μM), and butylated hydroxyanisole (Sigma, 10 μM). Cell death was measured by the WST-1 assay (Roche Applied Science).

Immunoprecipitations—RIP1 was immunoprecipitated from RBC sonicates with 10 μg of anti-RIP MAb (clone G322–2, BD Biosciences) overnight at 4 °C. Protein G Plus-agarose beads (Pierce) were added for 2 h at room temperature. Immunoprecipitates were washed, suspended in 1 × NuPAGE LDS buffer (Invitrogen), boiled, and run on SDS-PAGE followed by transfer to PVDF. VLY and ILY treatments were done with 0.5 hemolytic unit of each PFT for 30 min at 37 °C prior to immunoprecipitation.

Immunoblots—RBC lysates were obtained by sonication of 5% RBCs. Lysates from U937 and Jurkat cells were obtained by treatment of 4 × 106 cells in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, pH 7.5) on ice for 30 min. Lysate was cleared of nuclear material by centrifugation for 15 min at 4 °C. Cells were treated with 20 ng/ml of TNF-α for 2 h or 100 ng/ml of FasL for 5.5 h with Z-VAD-fmk. Lysates were probed on immunoblot by mAbs against the following proteins: RIP1, RIP3 (Cell Signaling Technology), MLKL (Cell Signaling Technology), hemoglobin (Thermo Scientific), and GAPDH (Cell Signaling Technology). Blots were developed using chemiluminescence.

RNAi—U937 monocytes were transfected with control or siRNA against RIP1 (Life Technologies) using the Genome-ONE-Neo EX HVJ envelope vector kit (Axxora) per the manufacturer’s instructions.

Quantitative PCR—RNA was extracted using a GenJet RNA purification kit (Thermo) per the manufacturer’s instructions. RNA was quantified by spectrophotometry. Quantitative PCR was done with an Applied Biosystems StepOne Plus thermocycler using Path-ID Multiplex One-Step RT-PCR kit (Life Technologies) and TaqMan Gene Expression Assays (Applied Biosystems) per the manufacturer’s instructions. The RNA concentration was normalized to 200 ng for each reaction. Specific TaqMan probes (Applied Biosystems) were: RIPK1 (Hs00169407_m1 FAM-MGB), RIPK3 (Hs01011177_g1 FAM-MGB), and MLKL (Hs04188508_m1 FAM-MGB). Reactions were performed in triplicate and relative quantification of expression was determined after normalizing with endogenous control GAPDH (Hs03929097_g1VIC-MGB). Fold-change was found using the ΔΔCt method of analysis.

In Vivo Brain Hypoxia-Ischemia Model—We induced hyperglycemia in neonatal (p10) mice subjected to regional HI brain injury. All studies were conducted according to a protocol approved by the Columbia University Institutional Animal Care and Use Committee (IACUC) and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. HI brain injury was induced as described (34, 35). Briefly, following permanent ligation of the right common carotid artery under 2% isoflurane anesthesia, mice were exposed to hypoxic (humidified 8% O2, 92% N2, Tech Air Inc., NY) insult for 15 min, at 37 ± 0.5 °C. A reperfusion was achieved by re-oxygenation in room air. Hyperglycemia was achieved by administration of 20% dextrose (10 μl/g intraperitoneal), 60 min prior to and immediately after hypoxia. At 60 min of reperfusion, circulating glucose levels were measured by tail bleed using a Blood Glucose Monitoring System, “One-Touch, Verio,” and expressed in mg/dl. There were three groups of mice: group 1: euglycemic HI mice received vehicle (4% DMSO in 0.9% normal saline); group 2: hyperglycemic HI mice received 2 mg/g of glucose in 20% dextrose, intraperitoneal and mixed with 4% DMSO in 0.9% normal saline; and group 3:
hyperglycemic HI mice treated with nec-1s. This group of mice received necrostatin (8.88 μg/g dissolved in 4% DMSO and 0.9% normal saline). The dose of nec-1s was calculated based on the report by Degterev et al. (4), in which nec-1s was injected intracerebroventriculatly at 2 μl/dose × 2 of 4 mm stock solution. Given the M₄ of nec-1s (277.7), the total dose of nec-1s in this report was 2.22 μg/dose × 2 doses = 4.44 μg/animal. In our experiments we injected nec-1s not intracerebroventricularly but by immunoprecipitation. Therefore, we arbitrarily increased the dose/animal by 10-fold such that the actual dose was 44.4 μg/animal in 20 μl of 4% DMSO. At 24 h of reperfusion all mice were sacrificed and the extent of brain injury was estimated using triphenly-tetrazolium staining. Infarct volume was expressed as % of the hemisphere ipsilateral to the carotid artery ligation side.

Statistics—Statistical analysis was performed using GraphPad Prism. Two-way analysis of variance with Bonferroni post test was used for all in vitro death assays and quantitative PCR. One-way analysis of variance test with Fisher’s post hoc analysis was used to determine the difference in cerebral infarct volume and glucose levels in vivo. All results are from 3 or more independent experiments.

Results

RBC Necroptosis Induced by Human-specific Bacterial PFTs Is Enhanced following Exposure to Hyperglycemic Levels of Glucose—To test the hypothesis that high levels of glucose prime cells for necroptosis, we first used the model of RBC necroptosis, which we have defined previously (5, 6). RBC necroptosis was induced by VLY or ILY and measured for primary RBCs pre-exposed to glucose concentrations ranging from 5 to 100 mM. RBC death by VLY or ILY increased in a dose-dependent manner with respect to different glucose levels (Fig. 1, A and B). RBC death by the hCD59-independent PFT, pneumolysin, which does not cause RBC necroptosis (5, 6), was not enhanced by exposure to high levels of glucose (Fig. 1C) but, rather, was inhibited, consistent with previous results on osmotic hemolysis (13, 14). The increase in RBC death by VLY and ILY as a result of exposure to high glucose levels was due to enhanced RBC necroptosis as inhibition of RIP1 with necrostatin-1s (nec-1s) (4) prevented it (Fig. 1, D and E).

Hyperglycemic Priming of RBC Necroptosis Depends on AGEs—We tested if RIP1 played a role in hyperglycemic enhancement of RBC necroptosis but there was no difference in total RIP1 protein levels or p-RIP1 following treatment with high levels of glucose (Fig. 2, A and B). Glycolysis was critical for the increase in RBC necroptosis as exposure to high levels of non-metabolizable 2-deoxyglucose had no effect on death (Fig. 2, C and D). Production of AGEs and ROS downstream of RIP1 depends on glycolysis (1, 5) and, indeed, enhancement of RBC necroptosis by glucose depended on AGEs (Fig. 2, E and F). Generation of ceramide by acid sphingomyelinase (aSMase), which is unrelated to glycolysis, or iron-dependent ROS are both important for RBC necroptosis as well. Although inhibition of these effectors resulted in a slight inhibition of RBC death (Fig. 2, G–J) it did not completely inhibit enhanced RBC death under hyperglycemic conditions such as that seen with inhibition of AGEs (Fig. 2, E and F). Therefore, these effectors appear to play little, if any, role in the hyperglycemic enhancement of RBC necroptosis.

Eryptosis Is Not Enhanced by Hyperglycemic Levels of Glucose—Eryptosis is a PCD unique to RBCs (15). It is induced by different stimuli, including hyperosmotic stress and hypercalcemia, and depends on p38 MAP kinase (15). As eryptosis is the only known PCD other than necroptosis in RBCs, we tested if exposure to high levels of glucose enhanced eryptosis. Eryptosis induced by hyperosmotic stress or excess Ca²⁺ decreased following treatment with high levels of glucose (Fig. 3) consistent with previous work showing that glucose depletion enhances this PCD (16). This indicates that the hyperglycemic priming of RBC death is specific to necroptosis.

RIP1-dependent Necroptosis of Jurkat and U937 Cells Is Enhanced following Exposure to Hyperglycemic Levels of Glucose—To determine whether the hyperglycemic priming of necroptosis was a shared phenomenon in nucleated cell types, we used U937 monocytes and Jurkat T cells, models of TNF-α and FasL necroptosis, respectively (4). U937 and Jurkat cells are maintained by passage in RPMI 1640 medium and thus are tolerant of a glucose level of ~10 mM (11.1 mM), beyond the physiological norm of 5 mM. Therefore, to test the effect of glucose levels equivalent to critical hyperglycemic levels

![FIGURE 1. Exposure to high levels of glucose primes human RBCs for necroptosis in vitro.](image-url)
Hyperglycemic priming of RBCs for necroptosis depends on glycolysis and AGEs.

A, immunoblot analysis of lysates from RBCs pre-incubated in 5 or 50 mM glucose (5, 50) and stimulated with VLY or ILY. RIP1 levels do not change in hyperglycemic conditions (50) relative to normal glucose (5).

B, immunoblots from RIP1 immunoprecipitations (IP) showing that RIP1 phosphorylation (p-RIP1) does not change in hyperglycemic conditions.

C and D, hemolysis assays of RBCs showing that treatment with 2-deoxyglucose has no effect on RBC death by VLY or ILY. E and F, enhanced hemolysis by VLY or ILY following exposure to high glucose levels is prevented by inhibition of AGE formation with pyridoxamine.

G and H, inhibition of iron-dependent ROS with 2,2-bipyridyl; or I and J, aSMase with desipramine (DPA) slightly inhibits RBC necroptosis but does not prevent hyperglycemia-enhanced necroptosis to the level of inhibition of AGE. Veh = vehicle. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
(25 mM), we used five times this amount (50 mM) and compared it with the effect of normal medium. Necroptosis was induced by TNF-α or FasL in the context of pan-caspase inhibition with Z-VAD-fmk (1, 4). Exposure to high levels of glucose enhanced cell death at several different lytic doses of TNF-α or FasL in both cell types (Fig. 4, A and B). The enhanced death was confirmed as RIP1-dependent necroptosis as it was inhibited by nec-1s (Fig. 4, C and D). Additionally, inhibition of RIP1 with specific siRNA (Fig. 4E) completely prevented the enhanced death of U937 cells (Fig. 4F). These results demonstrate that high levels of glucose prime U937 monocytes and Jurkat T cells for necroptosis.

Hyperglycemia Promotes Necroptosis Depends on AGEs and ROS Downstream of Glycolysis in Nucleated Cells

RIP1, RIP3, and MLKL Protein Levels Increase during Necroptosis Following Exposure to Hyperglycemia—Following hyperglycemic pre-treatment and stimulation of necroptosis in U937 or Jurkat cells, there was a robust increase in protein levels of RIP1 (Fig. 6A). This increase only occurred upon necrotic stimulation following exposure to high levels of glucose as RIP1 levels remained the same in the absence of necrototic stimulation (Fig. 6B). Additionally, RIP3 and MLKL protein levels increased during hyperglycemia-primed necroptosis (Fig. 6C). These increases were not due to transcription as mRNA levels of RIP1, RIP3, and MLKL were unchanged during hyperglycemia-primed necroptosis (Fig. 6D). These results suggest the possible activation of a positive feedback loop as a contributing factor in hyperglycemic enhancement of necroptosis. This also represents a fundamental difference in the hyperglycemic priming of necroptosis in nucleated cell types versus anucleate RBCs.

Exposure to Hyperglycemic Levels of Glucose Inhibits Extrinsic Apoptosis in Jurkat and U937 Cells—To determine whether enhancement of cell death by hyperglycemia was shared by other PCD pathways, we induced extrinsic apoptosis in U937 and Jurkat cells with TNF-α and FasL, respectively, the same...
stimuli used to induce necroptosis. In contrast to necroptosis, however, apoptosis was inhibited by exposure to high levels of glucose (Fig. 7). Moreover, treatment with high levels of glucose resulted in the failure to prevent cell death through pan-caspase inhibition (Fig. 7, C and D). This suggests that hyperglycemia inhibits extrinsic apoptosis and promotes caspase-independent PCD in its place. Additionally, protein levels of the necroptosis kinases, RIP1, RIP3, and MLKL increased following hyperglycemic pretreatment during apoptotic stimulation with TNF-α/H9251 (Fig. 7, E and F).

Hyperglycemia Exacerbates HI Brain Injury via RIP1-dependent Mechanism—Due to the fact that necroptosis drives damage in ischemic brain injury (3, 4) and hyperglycemia is linked to poor outcomes in such injuries (30, 31), we used an in vivo murine model of brain HI injury to determine whether necroptosis worsens the outcome of this injury during hyperglycemia. Compared with euglycemic littermates, hyperglycemic mice exhibited a greater extent of cerebral injury, as measured by infarct size, following HI insult (Fig. 8). Pre- and post-treatment with nec-1s prevented hyperglycemia-triggered exacerbation of HI brain injury. This suggests that hyperglycemia promotes necroptosis in the brain induced by HI insult. These data expand the relevance of the hyperglycemic priming of necroptosis to an in vivo model of necrotic injury.

FIGURE 5. Hyperglycemic priming of necroptosis depends on glucose metabolism, AGEs, and ROS. Death assays showing that enhanced necroptosis of U937 monocytes by TNF-α/Z-VAD (A) and Jurkat T cells by FasL/Z-VAD (B) following exposure to high levels of glucose is prevented by inhibition of glucose metabolism with 2-deoxyglucose (DG). Inhibition of cell death by 2-deoxyglucose is reversed by the addition of sodium pyruvate (pyruv). Enhanced necroptosis by TNF-α/Z-VAD (C) and FasL/Z-VAD (D) following treatment with high levels of glucose is partially prevented by inhibition of ROS with antioxidant butylated hydroxyanisole or inhibition of AGE formation with pyridoxamine relative to vehicle controls. E and F, cell death assays showing that enhancement of necroptosis by high glucose is not affected by inhibition of aSMase, which produces ceramide during necroptosis, with desipramine (inhibitor). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 6. RIP1, RIP3, and MLKL levels increase in cells during hyperglycemia-primed necroptosis. A, immunoblots of lysates from U937 monocytes stimulated with TNF-α/Z-VAD (zV) or Jurkat T cells stimulated with FasL/Z-VAD showing that RIP1 levels increase following pre-treatment with 50 mM glucose (50) in vitro. B, immunoblots of lysates from unstimulated U937 or Jurkat cells showing that RIP1 levels do not change following hyperglycemic pre-treatment relative to 10 mM glucose (10) in vitro. C, immunoblots showing that RIP3 and MLKL also increase during the hyperglycemic priming of necroptosis. D, mRNA transcripts of RIP1, RIP3, and MLKL are unchanged during hyperglycemic priming of necroptosis.

FIGURE 7. Hyperglycemic priming of death in U937 and Jurkat cells is specific to necroptosis and inhibits extrinsic apoptosis. Cell death of U937 and Jurkat cells by the extrinsic apoptosis stimuli TNF-α (A) and FasL (B) is inhibited following exposure to high levels of glucose. C, cell death that occurs in response to the apoptotic stimuli TNF-α and FasL cannot be prevented by pan-caspase inhibition with Z-VAD-fmk in hyperglycemic conditions. E, Western blots showing that stimulation of U937 monocytes by the apoptotic stimulus TNF-α (in the absence of Z-VAD) results in an increase in protein levels of RIP1, RIP3, and MLKL following hyperglycemic pretreatment. 10 = 10 mM glucose; 50 = 50 mM glucose. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Caspase-8 antagonizes necroptosis by promoting cleavage of RIP1 and RIP3 (17), which may implicate inhibition of this protease as a reason for increased levels of these kinases. However, this occurred in the context of Z-VAD-fmk, which strongly inhibits caspase-8 (18) making this less likely. RIP1 is subject to proteasome degradation via K48-linked polyubiquitylation promoted by A20 (19). As hyperglycemia induces degradation of A20 (20) this may account for elevated RIP1 levels. The increased RIP1 may result in increased recruitment of RIP3 and MLKL (2). This may result in a stronger necrosome as this complex is of amyloid structure (21). Increased formation of amyloid necrosomes in hyperglycemic conditions may account for the increased levels of RIP1, RIP3, and MLKL as amyloids are resistant to degradation (21). This work serves as an important starting point prompting further investigations into the mechanistic details of the hyperglycemic priming of necroptosis.

Hyperglycemia: a Condition That Specifically Enhances/Favors Necroptosis—An important finding in this work is that hyperglycemic enhancement of PCD is specific to necroptosis (Figs. 3 and 7). In fact, we demonstrated that high levels of glucose inhibit extrinsic apoptosis. This is consistent with previous work that showed that glucose uptake inhibits apoptosis, whereas glucose deprivation promotes it (22–26). Although extrinsic apoptosis was inhibited by exposure to high levels of glucose, significant amounts of caspase-independent death still occurred. This is evidenced by the findings that although the pan-caspase inhibitor, Z-VAD, inhibits apoptosis by TNF or FasL under normal glucose conditions, it fails to do so under hyperglycemic conditions (Fig. 7, C and D). As necroptosis and apoptosis share induction by the same ligands (1), it is tempting to speculate that hyperglycemia potentiates a shift from apoptosis to necroptosis. Lending support to this idea is the observation that levels of RIP1, RIP3, and MLKL increase following exposure to these apoptotic ligands under hyperglycemic conditions, in the absence of Z-VAD (Fig. 7E). This may create a situation in which levels of these kinases outweigh caspasases, shifting the balance of cell death to necroptosis. Additionally, glucose uptake and metabolism inhibits apoptosis by causing an increase in cFLIP, the endogenous inhibitor of caspase-8 (22–24). Moreover, increased induction of ROS due to hyperglycemia may result in the direct inactivation of caspase-8 (27). A potential shift from apoptosis to necroptosis may involve both an increase in necrototic kinase levels and inhibition of caspase-8. If this is the case, further work could provide mechanistic details that would reveal diabetes or stress hyperglycemia as conditions in which the balance of cell death is shifted from apoptotic toward necrototic cell death. It should be stressed, however, that additional work is necessary to definitively link hyperglycemia to PCD shift.

Translational Relevance of Hyperglycemic Priming of Necroptosis—Our in vivo data underscore a paramount significance of hyperglycemia-enhanced necroptosis, as we have found that hyperglycemia significantly exacerbates HI brain injury and nec-1s prevents this effect (Fig. 8). Our data are consistent with reports (28, 29) that found a potentially detrimental role of hyperglycemia in newborn piglets and rats subjected to HI insult. Given that early hyperglycemia (blood glucose > 150 mg/dl) during reperfusion following successful neonatal re-

**FIGURE 8. Hyperglycemia exacerbes hypoxia-ischemia brain injury via enhanced RIP1-dependent necroptosis.** Necrotic infarct size in mice subjected to brain hypoxia-ischemia was exacerbated significantly in hyperglycemic mice following 24 h of reperfusion. Exacerbation of infarct size by hyperglycemia was completely prevented by inhibition of RIP1 with nec-1s. Lower: corresponding, representative images of brain tissue stained with tetrazolium chloride.

**Discussion**

Hyperglycemic Priming of RBC Necroptosis Versus Necroptosis of Nucleated Cells—This work produced the novel finding that exposure to hyperglycemic levels of glucose primes cells for RIP1-dependent necroptosis (Figs. 1 and 4). This phenomenon was shared by three different types of blood cells: RBCs, monocytes, and T cells. Consistent with its role in necroptosis (1, 5), glycolysis was critical for hyperglycemic enhancement of necroptosis in RBCs, monocytes, and T cells (Figs. 2, C and D, and 5, A and B). In primary RBCs, AGEs were critical for this (Fig. 2, E and F), whereas iron-dependent ROS and aSMase, two other effectors of necroptosis (5), had little or no role (Fig. 2, G–J). Hyperglycemic enhancement of necroptosis in U937 monocytes and Jurkat T cells depended on ROS in addition to AGEs (Fig. 5, C and D) and was associated with a robust increase in protein levels of RIP1, RIP3, and MLKL (Fig. 6, A–C). This shows that although priming of necroptosis by hyperglycemia is shared by anucleated RBCs and nucleated cell types, it proceeds by different biochemical mechanisms. Importantly, this work demonstrates an overlooked connection between hyperglycemia and necroptosis. As we have firmly established this connection, it prompts deeper investigations into the underlying mechanism of hyperglycemic priming of necroptosis.

**Basis for Increased Protein Levels of RIP1, RIP3, and MLKL during Hyperglycemic Priming of Necroptosis**—The basis for the increase in levels of RIP1, RIP3, and MLKL (Figs. 6, A–C, and 7E) during the hyperglycemic priming of necroptosis is unclear. This does not appear to be due to enhanced transcription as mRNA levels of RIP1, RIP3, and MLKL do not change during hyperglycemia-enhanced necroptosis (Fig. 6D). Thus, these proteins may increase due to a post-translational mechanism.
Hyperglycemia Promotes Necroptosis

Suscitation is associated with poor neurological outcome in asphyxiated term newborns (30, 31), our findings carry a critical translational and mechanistic message: enhancement of necroptosis accounts for hyperglycemic exacerbation of HI brain injury. We also speculate that a hyperglycemia-induced shift from apoptotic cell death toward RIP1-dependent necroptosis contributes to exacerbated ischemic damage during hyperglycemia. However, this needs to be explored further. Indeed, necroptotic mechanisms of neuronal demise participate in the natural evolution of HI brain injury (3). The importance of the necroptosis-linked mechanism of neurodegeneration primed by hyperglycemia cannot be overestimated, as hyperglycemia is a known side effect of brain cooling (32), the only known effective neuroprotective strategy available for infants with HI brain injury. The groundwork laid in this study provides the basis for future studies that will investigate these translational possibilities and underlying mechanisms. Collectively, this work suggests that diabetes may be a condition in which cells are specifically primed to undergo necroptosis and may provide an explanation for exacerbation of diabetes-associated pathologies such as ischemia-reperfusion injury.

Author Contributions—T. J. L. conceived and coordinated the study, carried out experiments, interpreted results, and wrote the paper. S. S. performed experiments shown in Fig. 8. N. S. performed experiments shown in Fig. 7. V. S. T. designed, performed, and analyzed experiments shown in Fig. 8. A. J. R. interpreted results. T. J. L., N. S., V. S. T., and A. J. R. reviewed the results, revised the manuscript, and approved the final version of the manuscript.

References
1. Vandenabeele, P., Galluzzi, L., Vanden Bergh, T., and Kroemer, G. (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat. Rev. Mol. Cell Biol. 11, 704–714
2. Linkermann, A., and Green, D. R. (2014) Necroptosis. N. Engl. J. Med. 370, 455–465
3. Linkermann, A., Hackl, M. J., Kunzendorf, U., Walczak, H., Krautwald, S., and Jevnikar, A. M. (2013) Necroptosis in immunity and ischemia-reperfusion injury. Am. J. Transplant. 13, 2797–2804
4. Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G. D., Mitchison, T. J., Moskwitz, M. A., and Yuan, J. (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat. Chem. Biol. 1, 112–119
5. LaRocca, T. J., Stivison, E. A., Hod, E. A., Spitalnik, S. L., Cowan, P. J., Randis, T. M., and Ratner, A. J. (2014) Human-specific bacterial pore-forming toxins induce programmed necrosis in erythrocytes. MBio 5, e01251–14
6. LaRocca, T. J., Stivison, E. A., Mal-Sarkar, T., Hooven, T. A., Hod, E. A., Spitalnik, S. L., and Ratner, A. J. (2015) CD59 signaling and membrane pores drive Syk-dependent erythrocyte necroptosis. Cell Death Dis. 6, e1723
7. Fulda, S. (2013) Alternative cell death pathways and cell metabolism. Int. J. Cell Biol. 2013, 463637
8. Brownlee, M. (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414, 813–820
9. Singh, R., Barden, A., Mori, T., and Belin, L. (2001) Advanced glycation end-products: a review. Diabetes Metab. 44, 129–146
10. Wallace, D. C. (1999) Mitochondrial diseases in man and mouse. Science 283, 1482–1488
11. Brown, D. I., and Griendling, K. K. (2009) Nox proteins in signal transduction. Free Radic. Biol. Med. 47, 1239–1253
12. Sonksen, P. H. (2001) Insulin, growth hormone and sport. J. Endocrinol. 170, 13–25
13. Tato, L., and Rubaltelli, F. F. (1973) The protective action of glucose and nucleotides against hemolysis induced by menadione and phospholipase A. Clin. Chim. Acta 49, 349–352
14. Rotruck, J., Hoekstra, W., and Pope, A. (1971) Glucose-dependent protection by dietary selenium against haemolysis of rat erythrocytes in vitro. Nat. New Biol. 231, 223–224
15. Lang, E., Qadri, S. M., and Lang, F. (2012) Killing me softly: suicidal erythrocyte death. Int. J. Biochem. Cell Biol. 44, 1236–1243
16. Klarl, B. A., Lang, P. A., Kempe, D. S., Niemoeller, O. M., Akel, A., Sobiesiak, M., Eisele, K., Podolski, M., Huber, S. M., Wieder, T., and Lang, F. (2006) Protein kinase C mediates erythrocyte “programmed cell death” following glucose depletion. Am. J. Physiol. Cell Physiol. 290, C244–C253
17. O’Donnell, M. A., Perez-Jimenez, E., Oberst, A., Ng, A., Massoumi, R., Xavier, R., Green, D. R., and Ting, A. T. (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat. Cell Biol. 13, 1437–1442
18. Chauvier, D., Anki, S., Charriott-Marlange, C., Casimir, R., and Jacotot, E. (2007) Broad-spectrum caspase inhibitors: from myth to reality? Cell Death Differ. 14, 387–391
19. Bernassola, F., Ciechanover, A., and Melino, G. (2010) The ubiquitin proteasome system and its involvement in cell death pathways. Cell Death Differ. 17, 1–3
20. Shrikhande, G. V., Scali, S. T., da Silva, C. G., Damrauer, S. M., Csizmadia, E., Putnieti, P., Matthey, M., Arjoon, R., Patel, R., Siracuse, J. J., Maccariello, E. R., Andersen, N. D., Monahan, T., Peterson, C., Essayagh, S., et al. (2010) O-Glycosylation regulates ubiquitination and degradation of the anti-inflammatory protein A20 to accelerate atherosclerosis in diabetic ApoE-null mice. PLoS ONE 5, e14240
21. Li, J., McQuade, T., Siemens, B., Bapetschnig, J., Moriwaki, K., Hsiao, Y. S., Damko, E., Moquin, D., Walz, T., McDermott, A., Chan, F. K., and Wu, H. (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 150, 339–350
22. Nam, S. Y., Amoscatto, A. A., and Lee, Y. J. (2002) Low glucose-enhanced TRAIL cytotoxicity is mediated through the ceramide-Akt-FLIP pathway. Oncogene 21, 337–346
23. Muñoz-Pinedo, C., Ruiz-Ruiz, C., Ruiz de Almodóvar, C., Palacios, C., and López-Rivas, A. (2003) Inhibition of glucose metabolism sensitizes tumor cells to death receptor-triggered apoptosis through enhancement of death-inducing signaling complex formation and apical procaspase-8 processing. J. Biol. Chem. 278, 12759–12768
24. Vesely, E. D., Heilig, C. W., and Brosius, F. C., 3rd. (2009) GLUT1-induced cFLIP expression promotes proliferation and prevents apoptosis in vascular smooth muscle cells. Am. J. Physiol. Cell Physiol. 297, C759–C765
25. Cara-Maldonado, A., Tait, S. W., Ramírez-Peinado, S., Ricci, J. E., Fabregat, I., Green, D. R., and Muñoz-Pinedo, C. (2010) Glucose deprivation induces an atypical form of apoptosis mediated by caspase-8 in Bax-, Bak-deficient cells. Cell Death Differ. 17, 1335–1344
26. Li, H., Télémaque, S., Miller, R. E., and Marsh, J. D. (2005) High glucose inhibits apoptosis induced by serum deprivation in vascular smooth muscle cells via upregulation of Bcl-2 and Bcl-xl. Diabetes 54, 540–545
27. Borutaite, V., and Brown, G. C. (2001) Caspases are reversibly inactivated by hydrogen peroxide. FEBS Lett. 500, 114–118
28. Sheldon, R. A., Partridge, J. C., and Ferriero, D. M. (1992) Postischemic hyperglycemia is not protective to the neonatal rat brain. Pediatr. Res. 32, 489–493
29. Chang, Y. S., Park, W. S., Lee, M., Kim, K. S., Shin, S. M., and Choi, J. H. (1998) Effect of hyperglycemia on brain cell membrane function and energy metabolism during hypoxia-ischemia in newborn piglets. Brain Res. 798, 271–280
30. Spies, E. E., Lababidi, S. L., and McBride, M. C. (2014) Early hyperglycemia is associated with poor gross motor outcome in asphyxiated term newborns. Pediatr. Neurol. 50, 586–590
31. Basu, S. K., Kaiser, J. R., Guffey, D., Minard, C. G., Guillett, R., and Gunn, A. J. (2016) Hypoglycaemia and hyperglycaemia are associated with unfa...
vourable outcome in infants with hypoxic ischaemic encephalopathy: a post hoc analysis of the CoolCap Study. Arch. Dis. Child Fetal Neonatal Ed. 101, 149–155

32. Darwazeh, R., and Yan, Y. (2013) Mild hypothermia as a treatment for central nervous system injuries: positive or negative effects. Neural. Regen. Res. 8, 2677–2686

33. Gelber, S. E., Aguilar, J. L., Lewis, K. L., and Ratner, A. J. (2008) Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolytin from Gardnerella vaginalis. J. Bacteriol. 190, 3896–3903

34. Sosunov, S. A., Ameer, X., Niatsetskaya, Z. V., Utkina-Sosunova, I., Ratner, V. I., and Ten, V. S. (2015) Isoflurane anesthesia initiated at the onset of reperfusion attenuates oxidative and hypoxic-ischemic brain injury. PLoS ONE 10, e0120456

35. Niatsetskaya, Z. V., Sosunov, S. A., and Matsuukevich, D., Utkina-Sosunova, I. V., Ratner, V. I., Starkov, A. A., and Ten, V. S. (2012) The oxygen free radicals originating from mitochondrial complex I contribute to oxidative brain injury following hypoxia-ischemia in neonatal mice. J. Neurosci. 32, 3235–3244