Oxygen-Glucose Deprivation Induces Inducible Nitric Oxide Synthase and Nitrotyrosine Expression in Cerebral Endothelial Cells

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Background and Purpose—The cerebral endothelial cells (ECs) are a primary target of hypoxic or ischemic brain insults. EC damage may contribute to postischemic secondary injury. Massive production of NO after inducible NO synthase (iNOS) expression has been implicated in cell death. This study aimed to characterize bovine cerebral EC death in relation to iNOS expression after oxygen-glucose deprivation (OGD) in vitro.

Methods—OGD in bovine cerebral ECs in culture was induced by deleting glucose in the medium and by incubating the cells in a temperature-controlled anaerobic chamber. The extent of cell death was assessed by trypan blue exclusion, MTT assay, and LDH release. ELISA, gel electrophoresis, and staining by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling were used to examine DNA fragmentation. The expression of iNOS mRNA and protein was detected by reverse transcription–polymerase chain reaction and Western blotting, respectively. Nitrotyrosine expression was confirmed with Western blot analysis and immunostaining.

Results—Bovine cerebral EC death was dependent on the duration of OGD and showed selected biochemical, morphological, and pharmacological features suggestive of apoptosis. OGD also induced the expression of iNOS mRNA and protein in bovine cerebral ECs. Increased expression of nitrotyrosine, the product formed by peroxynitrite reaction with proteins, was also detected after OGD. The involvement of iNOS in EC death was suggested by partial reduction of cell death by NO synthase inhibitors, including 1-NAME-(1-iminoethyl)ornithine and nitro-L-arginine, and an NO scavenger, the Fe²⁺-N-methyl-D-glucamine dithiocarbamate complex.

Conclusions—OGD-induced bovine cerebral EC death involves an apoptotic process. Induction of iNOS with subsequent peroxynitrite formation may contribute to bovine cerebral EC death caused by OGD. (Stroke. 2000;31:1744-1751.)

Key Words: apoptosis ■ blood-brain barrier ■ cerebral ischemia ■ free radicals ■ nitric oxide

The brain requires a continuous supply of oxygen and glucose to maintain normal function. Loss of this supply, even for a short duration, leads to irreversible brain injury, including degeneration of neurons and other cell elements. Cerebral endothelial cells (ECs) have been shown to be more resistant than neurons or glia to ischemic insult. However, cerebral endothelial injury or death after ischemia may exacerbate brain damage and contribute to postischemic secondary injury characterized by the breakdown of the blood-brain barrier, leading to increased vascular permeability and vasogenic brain edema. Endothelial injury enhances leukocyte adhesion and also serves as a source of oxygen-derived free radicals.

Various therapeutic strategies aimed at reducing EC activation and injury in the postischemic inflammatory reaction have been explored. However, the molecular mechanism of cerebral EC death after ischemia has not been systematically studied. NO is a major signaling molecule generated in various cell types in the brain, including cerebral ECs. NO has also been implicated in tissue damage under a number of experimental paradigms, including brain injury after cerebral ischemia. NO synthesis is catalyzed by NO synthase (NOS). Of the 3 known isoforms of NOS, NO generated by neuronal NOS contributes to ischemic neuronal death, whereas constitutively expressed endothelial NOS may serve to protect cerebral ECs from ischemic insult. In disease states, including infection and ischemia, a third isoform of NOS, namely, inducible NOS (iNOS) may be expressed and contributes to inflammatory processes and ischemic brain damage. In the brain, iNOS expression has been noted in the inflammatory cells, such as the polymorphonuclear cells that infiltrate the ischemic brain and glial elements, including...
microglia and astrocytes. Cerebral ECs are also capable of expressing iNOS and producing NO under inflammatory conditions. In general, massive NO production by iNOS as occurs in various pathological states is considered cytotoxic. However, NO derived from iNOS has recently been shown to be cytotoxic in a number of cell types. Whether iNOS expression plays any role in the ischemic death of cerebral ECs has not been studied previously. In the present study, we sought to explore morphological, biochemical, and pharmacological features and the molecular mechanism of ischemic cerebral EC death by use of an in vitro model based on oxygen-glucose deprivation (OGD) in primary cultures of bovine cerebral ECs. The in vitro system allowed the characterization of molecular events relevant to iNOS expression.

Materials and Methods

Bovine Cerebral EC Culture
Bovine cerebral ECs were prepared and characterized as previously described. Briefly, fresh bovine brains in ice-cold Hanks’ balanced salt solution (HBSS, GIBCO-BRL) with antibiotics were freed of meninges and superficial blood vessels. The gray matter was homogenized and filtered, and the resulting microvessel fraction was then sequentially digested with collagenase B (4 mg/mL) for 2 hours and collagenase/dispase (1 mg/mL, Boehringer-Mannheim) for 8 hours, followed by centrifugation in 40% Percoll solution. The second band containing microvessels was collected and washed before plating onto collagen-coated dishes. Bovine cerebral ECs migrating from vessels were pooled to form a culture of proliferating endothelial cells that were maintained in medium containing 10% FCS, heparin (0.5 mg/mL), and endothelial growth supplements (75 μg/mL, Sigma Chemical Co). Bovine cerebral ECs of passages 4 to 6 were used, and the molecular purity was determined by immunostaining with factor VIII and vimentin, which were uniformly positive for factor VIII and vimentin.

Combined OGD
Confluent bovine cerebral ECs were transferred into a temperature-controlled (37 ± 1°C) anaerobic chamber (Forma Scientific) containing a gas mixture composed of 5% CO₂, 10% H₂, 85% N₂, and 0.02% to 0.2% O₂. The culture medium was replaced and washed with 0.5 mL of preheated deoxygenated glucose-free HBSS 4 times and then kept in the same medium in the hypoxia chamber for 1 to 8 hours. In some experiments, bovine cerebral ECs that underwent OGD were returned to a normoxic incubator under 5% CO₂/95% air for 16 hours. At the end of OGD, DMEM and MTT (Sigma) reagent (0.5 mg/mL) were added for 4 hours, followed by lysis solution (10% SDS in 0.01N HCl) for 14 hours. Absorbance was read at 540 nm in a multiple reader.

MTT Assay
At the end of OGD, DMEM and MTT (Sigma) reagent (0.5 mg/mL) were added for 4 hours, followed by lysis solution (10% SDS in 0.01N HCl) for 14 hours. Absorbance was read at 540 nm in a multiple reader.

LDH Release
Bovine cerebral EC death was also quantitatively assessed by measuring the extent of LDH release into the medium after OGD for 1 to 8 hours. The amount of LDH released after bovine cerebral EC lysis by 0.5% Triton 100 constitutes 100% cell death or “full kill.” The extent of cell death was expressed as percentage of full kill.

Quantification of Cytoplasmic Histone-Associated DNA Fragments by ELISA
A prominent feature of apoptosis is DNA fragmentation. A Cell Death Detection ELISA kit (Boehringer-Mannheim) was used to quantitatively determine the levels of histone-associated DNA fragments, including mononucleosomes and oligonucleosomes after OGD. The assay was based on the sandwich-enzyme immunoassay principle with the use of mouse monoclonal antibodies directed at DNA and histone. This assay allows the determination of mononucleosome and oligonucleosome levels in the cell lysates. Increases in DNA fragmentation over control values were quantitatively determined by an enrichment factor based on the following formula: enrichment factor = milliunits of the treated sample/milliunits of the vehicle-treated sample, where milliunits = absorbance (10⁻³).

Assessment of DNA Fragmentation by Agarose Gel Electrophoresis
A DNA isolation kit from Promega (catalog No. A1120) was used for the extraction of DNA after OGD. The cells in 100-mm dishes were lysed by the addition of 1.5 mL cell lysis solution and treated with RNase A solution. Proteins were precipitated by a solution provided with the DNA isolation kit, and DNA was hydrated. The DNA samples (10 μg per lane) were electrophoresed at 75 V for 2 hours in 1.5% agarose gel containing 0.4 μg/mL ethidium bromide in a Tris-acetate buffer (0.4 mol/L Tris, 0.25 mol/L sodium acetate, and 0.22 mmol/L EDTA, pH 7.8). DNA was visualized through ultraviolet transillumination and photographed. The ladder consists of DNA fragments, which differ in multiples of 180 to 200 bp.

DAPI Staining
Bovine cerebral ECs on coverslips were incubated in 1 μg/mL of 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes), a fluorescent probe for DNA, for 10 minutes after fixation with 4% paraformaldehyde. After they were washed with PBS, the slides were examined under a fluorescence microscope.

TUNEL Staining
Confluent bovine cerebral ECs grown on coverslips were subjected to OGD for 8 hours, followed by fixation with 4% paraformaldehyde. Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining was performed according to the ONCOR kit protocol (catalog No. S7100-kit) as has been previously described.

RNA Isolation
RNA isolation has been previously described. In brief, total RNA from bovine cerebral ECs was prepared with the use of TRI reagent from Molecular Research Center, Inc. Cells were lysed and extracted by adding 1.0 mL of TRI reagent. The lysate was added to 100 μL of chloroform, and the solution was mixed and centrifuged. The supernatant was removed, mixed with an equal volume of isopropanol, and kept at 4°C for at least 90 minutes. After centrifugation at 14,000g for 30 minutes at 4°C, the pellet was washed with 75% ethanol and then centrifuged again for 10 minutes at 4°C. The RNA fraction was then resuspended in water. Total RNA was quantified by spectrophotometry.

RT-PCR
Reverse transcription (RT)–polymerase chain reaction (PCR) for iNOS has also been reported. Briefly, equal amounts of RNA (2 μg) were reverse-transcribed with oligo(dT) and 500 μmol/L dNTPs
(BRL Life Technologies, Inc.), 20 U Rasin (Promega), 200 μmol/L dithiothreitol, and 200 U reverse transcriptase (BRL) for 50 minutes at 42°C. After incubation, the sample was heated for 5 minutes at 95°C, diluted, and divided into aliquot portions. The PCR was performed with a reaction mixture containing cDNA transcribed from 50 ng RNA, 100 μmol/L dNTPs, 1 μmol/L of each primer, (α-32P)dCTP, 1.5 mmol/L MgCl₂, and 2.5 U Taq polymerase (BRL). PCR reaction conditions were as follows: 23 cycles (each cycle consisted of 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C) and a delay time of 10 minutes. The conditions were determined in preliminary studies to be within the linear range in terms of RT input and PCR cycles. The PCR products were electrophoresed through a 12% PAGE gel and visualized by PhosphorImager (Molecular Dynamics) quantification. The relative mRNA levels of each gene were determined after normalization on the base of endogenous cyclophilin mRNA. The PCR experiments were repeated 3 times each by using separate sets of cultures.

**Western Blot Analysis**

Detection of iNOS and nitrotyrosine expression in bovine cerebral ECs by Western blot analysis has been previously reported. Bovine cerebral ECs were homogenized by sonification in a Western blot buffer (10 mmol/L Tris-HCl containing 2 mmol/L EDTA, 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L iodoacetate, 5 mmol/L N-ethylmaleimide, and 0.1 U/mL aprotinin, pH 7.2) and centrifuged at 10,000g for 15 minutes. Twenty micrograms of protein from the supernatant of each sample was loaded onto an 8% polyacrylamide gel, resolved by SDS/PAGE, and transferred to nitrocellulose membranes by electrophoresis. For iNOS assay, the membranes were blocked in TBST buffer containing 20 mmol/L Tris-HCl, 5% nonfat milk, 150 mmol/L NaCl, and 0.05% Tween 20 (pH 7.5) for 1 hour at room temperature. For detection of nucleotides, the membrane was briefly washed 3 times with 100 mmol/L sodium dithionite, which was dissolved in 100 mmol/L nitrogen-bubbled sodium bicarbonate (pH 10) to eliminate nonspecific protein binding to nucleotide antibody, and then the membrane was washed with TBST as described above. Primary polyclonal antibody against a mouse macrophage iNOS (Transduction Laboratory) at a 1:500 dilution or monoclonal anti-nitrotyrosine antibody33 at a 1:2000 dilution was added to the membrane and incubated at 4°C overnight or for 2 hours at room temperature. The membranes were washed with TBST 3 times at 10-minute intervals, incubated with the second antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase [1:5000 for iNOS] and sheep anti-mouse IgG conjugated with horseradish peroxidase [1:7000 for nitrotyrosine]) at 37°C for 1 hour, and then washed 3 times each at 10-minute intervals with TBST and 2 times each for 2 minutes with TBS (TBST without Tween 20). The color reaction based on the Blot AP System was as described in the technical manual provided by Promega for iNOS expression or the chemiluminescence method as described in Amersham Life Science protocol RPN2106 for nitrotyrosine.

**Immunocytochemical Staining for Cytochrome c and Nitrotyrosine**

Bovine cerebral ECs were fixed with 4% paraformaldehyde and washed 3 times with PBS. For indirect immunofluorescence, the primary antibodies were monoclonal antibodies against cytochrome c (1:100, Pharmingen) and nitrotyrosine (1:100, Transduction Labs). The secondary antibody was goat anti-rabbit IgG conjugate/rhodamine34 (1:100, Promega). Negative controls were prepared with omission of the primary antibody. Additional negative control studies were conducted by incubation with the anti-nitrotyrosine antibody in the presence of exogenous nitrotyrosine (10 mmol/L, Transduction Labs).

**Treatment With Caspase and NOS Inhibitors**

In some experiments, the bovine cerebral ECs in 24-well plates were treated with 50 μmol/L of ZVAD-fmk (Enzyme Systems Products) before undergoing OGD for 8 hours. In a separate set of experiments, cells in 24-well plates were treated with 1 μmol/L of NOS inhibitors, L-N^G-(1-iminoethyl)ornithine (NIL) or nitro-L-arginine (L-NA), or an NO scavenger, the Fe^3+-N-methyl-d-glucamine dithiocarbamate complex [(MGD)_2-Fe^3+], before undergoing OGD for 8 hours. After the OGD treatment, the extent of cell death was assessed by the MTT assay as explained above.

**Statistical Analyses**

Quantitative data are expressed as mean ± SD on the basis of at least 2 separate experiments of triplicate samples. Difference among groups was statistically analyzed by 1-way ANOVA followed by the Bonferroni post hoc test. Comparison between 2 experimental groups was based on the 2-tailed t test. A value of P<0.05 was considered significant.

**Results**

**Time Course of OGD-Induced Bovine Cerebral EC Death**

Bovine cerebral ECs that underwent OGD for 1 hour showed little morphological change and sustained virtually no cell death. Changes in cell morphology occurred if OGD duration was 2 hours. The cell bodies became thin, elongated, and shrunken. The spaces between cells were greater in cells with OGD than in control cells (Figure 1). The extent of OGD-induced cell death was assessed by counting the number of living cells that exclude trypan blue (Figure 2A). The extent of cell death was ≈20%, ≈35%, and ≈40% after 4, 6, and 8 hours, respectively, of OGD. Similar time-dependent OGD was also noted on the basis of the MTT assay (Figure 2B) and LDH release (Figure 2C).

**OGD-Induced DNA Fragmentation and Cytochrome c Release**

We determined the extent of DNA fragmentation by quantitative assessment of cytoplasmic histone-associated DNA fragments. OGD gradually increased DNA fragmentation in a time-dependent manner (Figure 3A). DNA laddering was also evident after OGD exposure for ≥4 hours (Figure 3B). An increase in nuclear DNA strand breaks after OGD was confirmed morphologically by TUNEL staining (Figure 3C). Furthermore, DAPI staining showed cell nuclear condensation after OGD (Figure 4). Cytochrome c release into the cytosol was also noted after OGD (Figure 4). Together, these findings are compatible with the contention that OGD-induced bovine cerebral EC death may involve an apoptotic process.

**OGD-Induced iNOS Expression**

iNOS mRNA expression in bovine cerebral ECs was increased after OGD on the basis of RT-PCR. An increase in iNOS mRNA expression was detectable after OGD for ≥2 hours. Peak expression occurred after OGD for 6 hours (Figure 5A). iNOS expression at the protein level was confirmed by Western blot analysis (Figure 5B).

**Expression of Nitrotyrosine in Bovine Cerebral ECs During OGD**

NO and superoxide (O₂⁻) interact to form peroxynitrite (ONOO⁻). ONOO⁻ is a highly toxic reactive oxygen species, which in turn reacts with tyrosine in proteins to form nitrotyrosine, a stable oxidation product. Western blot analysis detected an increase in nitrotyrosine formation in...
bovine cerebral ECs after OGD. Nitrotyrosine expression increased with the duration of OGD. A single protein band showing nitrotyrosine immunoreactivity was 68 kDa (Figure 6). Nitrotyrosine immunoreactivity was also detected in bovine cerebral ECs after OGD by immunocytochemistry (Figure 7).

Effects of Caspase Inhibitor, NOS Inhibitors, and NO Scavenger on OGD-Induced Bovine Cerebral EC Death

zVAD-fmk, a broad-spectrum caspase inhibitor, was effective in reducing OGD-induced bovine cerebral EC death (Figure 8A). NOS inhibitors, including NIL, a selective iNOS inhibitor, and L-NA, a nonspecific NOS inhibitor, partially reduced bovine cerebral EC death. (MDG)2-Fe2+, an NO scavenger, was also effective (Figure 8B).

Discussion

Ischemic or hypoxic insults can cause cell death by both necrosis and apoptosis.37,38 We found that OGD-induced bovine cerebral EC death exhibited biochemical, morphological, and pharmacological features suggestive of apoptosis. DNA fragmentation in bovine cerebral ECs after OGD was noted on the basis of ELISA, TUNEL stain, and gel electrophoresis. Immunostaining showed cytochrome c release. Cytochrome c is a mitochondrial respiratory component that translocates to the cytosol and activates DEVD (Asp-Glu-Val-Asp)-specific caspases in cells dying of apoptosis.39 Although none of the findings described above is fully specific for apoptosis, the observation that zVAD-fmk, a broad-spectrum caspase inhibitor, could substantially increase the cell viability from 25% to 75% strengthens the role of apoptosis in OGD-induced bovine cerebral EC death.

Cerebral ECs have been shown to express iNOS and produce NO under inflammatory conditions.21,22 iNOS immunoreactivity has also been identified in cerebral ECs in the ischemic brain.17,40 Similarly, hypoxia enhances iNOS expression in cytokine-treated murine macrophages,41 in rat mesangial cells,42 and in the rat lung.43 In the present study, we noted that OGD induced iNOS mRNA and protein expression in bovine cerebral ECs on the basis of RT-PCR and Western blot. Expression of iNOS is associated with prolonged production of high levels of NO.23 Overproduction
of NO may exacerbate ischemic brain injury.\textsuperscript{10,11} NO, when produced in excess, reacts with $O_2$ to form ONOO$^-$, which has been proposed to play an important role in the cellular damage associated with the overproduction of NO.\textsuperscript{35} NO and $O_2$ may act synergistically to enhance ONOO$^-$-mediated toxicity in cerebral endothelial cells.\textsuperscript{44} ONOO$^-$ reacts with proteins and results in the oxidation of tryptophan and cysteine residues. This process also leads to the nitration of tyrosine, formation of dityrosine, and 2,4-dinitrophenyl-hydrazine–reactive carbonyls, leading to protein fragmentation.\textsuperscript{45} The formation of 3-nitrotyrosine represents a likely ONOO$^-$-mediated protein modification that may be different from modifications mediated by other reactive oxygen species.\textsuperscript{46} We found that OGD also enhanced nitrotyrosine expression at 4 hours and peaked at 8 hours. Nitrotyrosine was identified in a band with a molecular size of $\approx 68$ kDa. The particular protein species that is vulnerable to nitration remains to be identified. The expression of nitrotyrosine in bovine cerebral ECs after OGD was further confirmed by immunohistochemical studies. The observation that nitrotyrosine immunoreactivity could be blocked by exogenous nitrotyrosine suggests specificity of nitrotyrosine moiety in bovine cerebral ECs after OGD.

The role of NO in the regulation of apoptosis is complex. Although NO has been shown to induce apoptosis in several cell types, it is cytoprotective in others, depending on the particular biological conditions, the concentration and rate of NO production, and its redox state.\textsuperscript{23,47,48} NO-mediated apoptosis has been reported in macrophages,\textsuperscript{59} astrocytes,\textsuperscript{50} and PC12 cells.\textsuperscript{51} On the other hand, NO protects against apoptosis in neurons,\textsuperscript{52} hepatocytes,\textsuperscript{53} human umbilical venous cells,\textsuperscript{54} and B lymphocytes.\textsuperscript{55} The protective effect of NO could be either via cGMP-mediated interruption of apoptotic signaling, Bel 2 upregulation, or a direct inhibition of caspase activity.\textsuperscript{25,52,56–58} In contrast, the proapoptotic effects may be due to a mechanism involving excitotoxic mediators, Ca$^{2+}$ overload and the subsequent activation of caspases,\textsuperscript{59} proteosome inhibition leading to p53 accumulation,\textsuperscript{60} or increased...
Bax production. NO production after increased iNOS activity has also shown conflicting effects on apoptosis. Results derived from the present study revealed that the extent of bovine cerebral EC death with features suggestive of apoptosis was dependent on the duration of OGD and was correlated with the expression of iNOS. It is interesting to note that cerebral ECs were more resistant to OGD than were cortical neurons in culture. Neurons exposed to OGD for 1 hour showed extensive neuronal death over a period of 24 hours. In contrast, bovine cerebral ECs exposed to OGD for up to 2 hours showed virtually no delayed cell death during the same period. The in vitro findings are consistent with the in vivo observation showing that ECs are more resistant to focal cerebral ischemia than are neurons.

To further explore whether iNOS expression contributes to OGD-induced bovine cerebral EC death, we tested NOS inhibitors that are either nonspecific (L-NA) or iNOS selective (NIL). L-NA and NIL were effective in partially reducing OGD-induced death in bovine cerebral ECs. The notion that bovine cerebral EC death after OGD is mediated by NO is further suggested by the cytoprotective role of an NO scavenger, (MGD)Fe2.

In summary, results from the present study show that apoptosis may be involved in OGD-induced bovine cerebral EC death. The OGD death paradigm probably involves multiple mechanisms, with iNOS expression contributing to bovine cerebral EC death. Understanding the mechanism of cerebral EC death after OGD may aid in the development of therapeutic strategies to reduce secondary ischemic brain injury caused by postischemic hypoperfusion and blood-brain barrier dysfunction.
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Most of the effort in elucidating the cellular biological mechanisms of ischemic brain injury has appropriately focused on neurons. However, there is increasing evidence that ischemia-induced vascular damage is an integral step in the cascade of the cellular and molecular events initiated by cerebral ischemia. While endothelial cells express critical inflammatory mediators in response to ischemia, alterations in integrin expression and endothelial function may lead to increased vascular permeability, edema formation, and hemorrhage. However, little is known about the effects of hypoxia-ischemia on cerebral endothelial cells. In the accompanying article, Xu and colleagues investigated the mechanisms of endothelial injury in bovine cerebral endothelial cell cultures subjected to oxygen-glucose deprivation. They found that oxygen-glucose deprivation induces expression of inducible nitric oxide synthase (iNOS) in endothelial cells and leads to cell death by a mechanism resembling apoptosis. Pharmacological inhibition of iNOS attenuates endothelial cell death, which suggests that NO produced by iNOS, probably through peroxynitrite, its reaction product with superoxide, is involved in the killing. This is an important contribution that sheds light on a poorly understood, yet highly relevant aspect of the cellular biology of cerebral ischemia. The observation that endothelial cells undergo apoptosis is an exciting new finding that provides a previously unrecognized mechanism by which antiapoptotic agents, such as caspase inhibitors, may protect the ischemic brain. Furthermore, the observation that iNOS may be linked to endothelial apoptosis provides an insight into the pathogenic significance of the endothelial iNOS expression observed in models of cerebral ischemia and in human stroke as well. While large amounts of NO generated by iNOS could kill endothelial cells by an apoptotic mechanism, they could also produce endothelial cell dysfunction, resulting in vascular dysregulation and exacerbation of ischemia. The latter possibility is supported by recent findings that iNOS gene transfer to cerebral arteries blocks endothelium-dependent relaxation of the transfected vessel. Therefore, the findings of the present study suggest that vascular iNOS expression could contribute to brain injury by multiple mechanisms.

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