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Mitochondrial Dysfunction Induced by Nuclear Poly(ADP-Ribose) Polymerase-1: a Treatable Cause of Cell Death in Stroke

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Abstract

Many drugs targeting excitotoxic cell death have demonstrated robust neuroprotective effects in animal models of cerebral ischemia. However, these neuroprotective effects have almost universally required drug administration at relatively short time intervals after ischemia onset. This finding has translated to clinical trial results; interventions targeting excitotoxicity have had no demonstrable efficacy when initiated hours after ischemia onset, but beneficial effects have been reported with more rapid initiation. Consequently, there continues to be a need for interventions with efficacy at later time points after ischemia. Here, we focus on mitochondrial dysfunction as both a relatively late event in ischemic neuronal death and a recognized cause of delayed neuronal death. Activation of poly(ADP-ribose) polymerase-1 (PARP-1) is a primary cause of mitochondrial depolarization and subsequent mitochondria-triggered cell death in ischemia reperfusion. PARP-1 consumes cytosolic NAD^+, thereby blocking both glycolytic ATP production and delivery of glucose carbon to mitochondria for oxidative metabolism. However, ketone bodies such as pyruvate, beta- and gamma-hydroxybutyrate, and 1,4-butanediol can fuel mitochondrial metabolism in cells with depleted cytosolic NAD^+ as long as the mitochondria remain functional. Ketone bodies have repeatedly been shown to be highly effective in preventing cell death in animal models of ischemia, but a rigorous study of the time window of opportunity for this approach remains to be performed.

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Keywords

Ischemia; Mitochondrial depolarization; Mitochondrial permeability transition; Poly(ADP-ribose) polymerase-1; NAD^+; Apoptosis-inducing factor

Introduction

Ischemic stroke results from critically reduced blood flow in one or more arteries of the brain or spinal cord. If blood flow is not promptly restored, cells in the ischemic territory die of energy failure. If blood flow is restored ("ischemia reperfusion"), cells may nevertheless go on to die from excitotoxicity, effects of inflammation, or programmed cell death. These processes may also kill cells that are not themselves subjected to critical ischemia but are in the vicinity of ischemic tissue.

Of these injury processes, neuronal excitotoxicity is the one triggered most rapidly after ischemia. Excitotoxicity results from sustained action of glutamate at neuronal glutamate receptors, primarily NMDA-type glutamate receptors [1, 2]. In stroke, this is caused by the release of neurotransmitter glutamate, impaired glutamate reuptake, and reversed action of glutamate uptake transporters during ATP depletion [3, 4]. The importance of excitotoxicity as a cell death mechanism in stroke has been firmly established in a variety of stroke models and animal species, using pharmacological, genetic, and other approaches to block NMDA receptor activation [5-14]. Excitotoxicity is a particularly important cell death mechanism in stroke involving ischemia reperfusion [10, 15, 16]. Decades of work in this area have identified multiple steps in the excitotoxic cell death pathway, and Fig. 1 diagrams the relationships between several of these steps. Pharmacological and genetic interventions affecting each of these steps have demonstrated robust neuroprotective effects in animal models of brain ischemia, particularly ischemia reperfusion [17]. However, the animal models have also demonstrated a relatively short window of opportunity for these interventions, on the order of 30-90 min in most cases [17-19]. This short window of opportunity has, unfortunately, translated well to human clinical studies; interventions targeting excitotoxic injury have had no demonstrable efficacy when initiated many hours after ischemia onset, though more rapid initiation has shown beneficial effects [17, 20].

The continued need for interventions with efficacy at later time points after ischemia warrants a reassessment of events occurring later in the cascade displayed in Fig. 1. In this review, we focus on mitochondrial dysfunction, as both a relatively late event in ischemic neuronal death and a recognized cause of delayed neuronal death. We propose that available interventions targeting mitochondrial dysfunction may provide a more clinically applicable time window of opportunity.

Mitochondrial Dysfunction in Stroke

Mitochondria have been established as important components of ischemic neuronal death for several decades. Early studies using electron microscopy demonstrated swollen and calcium-laden mitochondria as one of the earliest manifestations of ischemic neuronal demise [21]. Subsequent studies identified several "effector" pathways by which mitochondrial

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dysfunction can lead to ischemic neuronal death [22, 23]. Additional evidence that mitochondrial demise is a causal event in ischemic neuronal death came from studies showing that ischemia reperfusion can lead to an initial near normalization of mitochondrial function, with a secondary deterioration occurring several hours later [24]. A similar and possibly related process has been identified in cultured neurons, in which NMDA receptor stimulation leads to transient mitochondrial calcium elevations, which only later results in delayed mitochondrial depolarization and cellular calcium deregulation [25, 26]. Together, this literature suggests that (a) ischemia and excitotoxicity lead to mitochondrial dysfunction, often in a delayed manner; that (b) mitochondrial dysfunction is a causal event in ischemic and excitotoxic neuronal death; and that (c) therapeutic interventions targeting mitochondrial dysfunction may be able to rescue neurons at delayed time points after ischemic or excitotoxic insults.

Mitochondria and Cell Fate

Mitochondria can induce cell death by at least three interrelated mechanisms: apoptosis, parthanatos, and energy failure. Release of cytochrome C from mitochondria triggers caspase-mediated apoptotic neuronal and glial death [27, 28]. Although these cell types do not display many of the morphological hallmarks of classical apoptosis [29-31], caspase inhibitors have been shown to reduce ischemic injury in certain settings, particularly in mild injury and in the immature brain [32, 33]. Release of apoptosis-inducing factor (AIF) from mitochondria induces neuronal cell death by a process that is biochemically and morphologically distinct from classical apoptosis [34]. This process, termed parthanatos, is not caspase dependent but, instead, involves DNA cleavage by endonuclease G [35, 36]. Both apoptosis and parthanatos are promoted by mitochondrial permeability transition (MPT) [37-39], which involves formation of a large-diameter pore spanning the inner and outer mitochondrial membranes. MPT, in turn, is promoted by mitochondrial depolarization, calcium loading, oxidative stress, glutathione depletion, and other factors [40, 41]. MPT also stops mitochondrial energy production and releases mitochondrial calcium into the cytosol. While transient MPT may have physiological functions, persistent or extensive MPT leads to cell death.

The primary function of mitochondria is to provide oxidative ATP production, and an additional way that mitochondria contribute to neuronal demise is by failing to generate ATP. Glucose normally provides more than 95 % of mitochondrial substrate to the brain, which is metabolized first in the cytosol to pyruvate (glycolysis) to generate two ATPs per glucose, and then in mitochondria to CO_2 to produce an additional 32-34 ATPs per glucose molecule (Fig. 2). Brain glucose is normally metabolized almost completely to CO_2, underlining the importance of oxidative mitochondrial ATP production to brain bioenergetics. Nevertheless, neurons and glia in brain can survive in the absence of oxygen for up to several hours by glycolytic ATP production alone [42-44]. This scenario is characteristic of the ischemic penumbra [45, 46]. By contrast, loss of both oxygen and glucose supply leads to near-complete ATP depletion within 3 min [47]. The brain, unlike most other tissues, relies almost exclusively on glucose for energy metabolism. Interruption of glycolysis in the brain, consequently, also produces complete energy failure (even with oxygen present), because this blocks both glycolytic ATP production and delivery of

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glucose carbon to the mitochondria (Fig. 2). As detailed below, extensive activation of poly(ADP-ribose) polymerase-1 by ischemia can indirectly produce a block in glycolysis which persists after restoration of blood flow. This can effectively “starve” mitochondria and can lead to delayed mitochondrial depolarization and induction of mitochondrial cell death pathways.

Poly(ADP-Ribose) Polymerase-1 in Ischemic Brain Injury

Poly(ADP-ribose) polymerase-1 (abbreviated as PARP-1) is an abundant nuclear enzyme that is activated by DNA strand breaks or kinks [48]. Activated PARP-1 hydrolyzes NAD^+ and transfers the ADP-ribose moieties to form branched chains of ADP-ribose on lysine residues of histones and other acceptor proteins [48]. PARP-1 normally functions to facilitate DNA repair and prevent chromosomal rearrangements [49], but extensive PARP-1 activation depletes the cytosolic NAD^+ pool and causes cell death [50]. In ischemia, sustained action of glutamate at neuronal NMDA receptors leads to the production of peroxynitrite and other reactive oxygen species that damage DNA and activate PARP-1 [51-54] (Fig. 1). This is an important cause of cell death in animal models of stroke, as evidenced by studies in which either PARP-1 inhibitors or PARP-1 gene deletion markedly improved outcome. These studies include animal models of focal ischemia [55-57], global ischemia [58, 59], hypoglycemia [60-62], and other conditions in which oxidative stress occurs [50, 63]. Unfortunately, like other neuroprotective interventions, PARP-1 inhibitors show a relatively short time window of efficacy in these animal models.

PARP-1 and Mitochondria

It was proposed more than 20 years ago that PARP-1 causes cell death by depletion of NAD^+ and resultant energy failure [64]. Importantly, cytosolic and mitochondrial NAD^+ pools are physically separated by the mitochondrial membranes, such that only the cytosolic pool is accessible to nuclear PARP-1 unless mitochondrial disruption or mitochondrial permeability transition occur [65, 66]. How then, can PARP-1 activation in the nucleus cause mitochondrial dysfunction? Cytosolic NAD^+ is required for the GAPDH step of glycolysis, and extensive PARP-1 activation can reduce cytosolic NAD^+ to levels preventing glucose utilization [67-70]. The resulting glycolytic blockade stops the flux of glucose-derived pyruvate and NADH to the mitochondria, thereby starving mitochondria of energy substrate and producing mitochondrial depolarization (Fig. 2).

A second way that PARP-1 activation can affect mitochondrial function is through effects of poly(ADP-ribose) cleavage products formed in the nucleus. These fragments can act directly on mitochondrial membranes to induce AIF release [71, 72], suggesting that PARP-1-induced AIF release may occur independent of NAD^+ depletion. However, studies performed in cultured neurons and astrocytes show that PARP-1-induced mitochondrial depolarization, AIF release, and cell death are all blocked by normalizing cytosolic NAD^+ levels and, further, that NAD^+ depletion alone can induce mitochondrial depolarization, AIF release, and cell death in the absence of PARP-1 activation [37, 73] (Figs. 3 and 4). These studies were performed in a medium containing only glucose as an energy substrate, in order to mimic conditions of brain extracellular fluid. However, standard culture medium contains

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substantial concentrations of amino acids and other substrates that do not require glycolysis or NAD^+ for entry into oxidative metabolism, and have been shown to rescue neurons and other cell types from PARP-1-induced cell death [37, 67, 69, 73]. Consequently, studies performed in a standard culture medium may mask the effects of PARP-1 that are mediated by cytosolic NAD^+ depletion.

Mitochondrial Resuscitation

Given that activated PARP-1 consumes cytosolic NAD^+, and NAD^+ is required for glycolysis, ischemia-induced PARP-1 activation may render cells unable to utilize glucose even when glucose availability is restored by reperfusion. However, cells in this state remain capable of metabolizing substrates such as pyruvate, which can be utilized by mitochondria without glycolysis (Fig. 2). Cell culture studies have confirmed that supplying cells with non-glucose substrates after PARP-1 activation will fuel mitochondrial oxidative metabolism and prevent mitochondrial depolarization, AIF release, and cell death [37, 68, 69, 73] (Figs. 3 and 4).

Can this approach be extended to rescue brain mitochondria in vivo? One issue is blood-brain barrier transit. The capacity for glucose flux across the blood-brain barrier is far higher than for any other metabolic substrates, and circulating levels of non-glucose substrates are normally insufficient to fuel brain metabolism. On the other hand, the movement of pyruvate and other metabolites into the brain is driven by mass action, and consequently, exogenous administration can greatly increase their flux across the blood-brain barrier. It has been shown, for example, that pyruvate administered to rats at a dose of 500 mg/kg prevented hippocampal neuronal death and preserved spatial memory after severe hypoglycemia [74]. This somewhat surprising result likely stems from the fact that the high blood levels achieved (estimated at 5 mM, 500 times higher than normal circulating levels of pyruvate) allowed significant brain penetration.

Pyruvate has also been shown to be neuroprotective in several species and models of brain ischemia, in an extensive literature that has been largely overlooked (Table 1). Most of these studies propose other mechanisms for the effects of pyruvate, such as free radical scavenging, suppression of NFκB signaling, and Ca^{2+} chelation. While these mechanisms are possible, it is noteworthy that not only pyruvate but also several other ketone bodies such as γ-hydroxybutyrate and 1,4-butanediol that also can be metabolized by mitochondria in the absence of cytosolic NAD^+ have also shown efficacy in animal models, of ischemia. Conversely, the administration of exogenous lactate, which does require cytosolic NAD^+ for mitochondrial metabolism, is not neuroprotective.

Conclusions

These arguments above do not constitute proof that pyruvate can provide “mitochondrial resuscitation” after ischemia, but they do suggest that this possibility merits examination. In particular, a crucial question that does not appear to have been rigorously addressed is how long after ischemia (or ischemia reperfusion) pyruvate administration can provide a neuroprotective effect. If pyruvate works by fueling “starved” mitochondria, then, this

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approach should, in principle, provide a longer time window of opportunity than more standard neuroprotective approaches, which act upstream of mitochondria. One study showed significant efficacy with onset as late as 12 h after ischemia reperfusion [75], but most studies have not evaluated time points past 3 h. Efficacy at even 3-6 h may be clinically useful as an adjunct to thrombolytic therapy, given the availability and safety of these metabolic intermediates.

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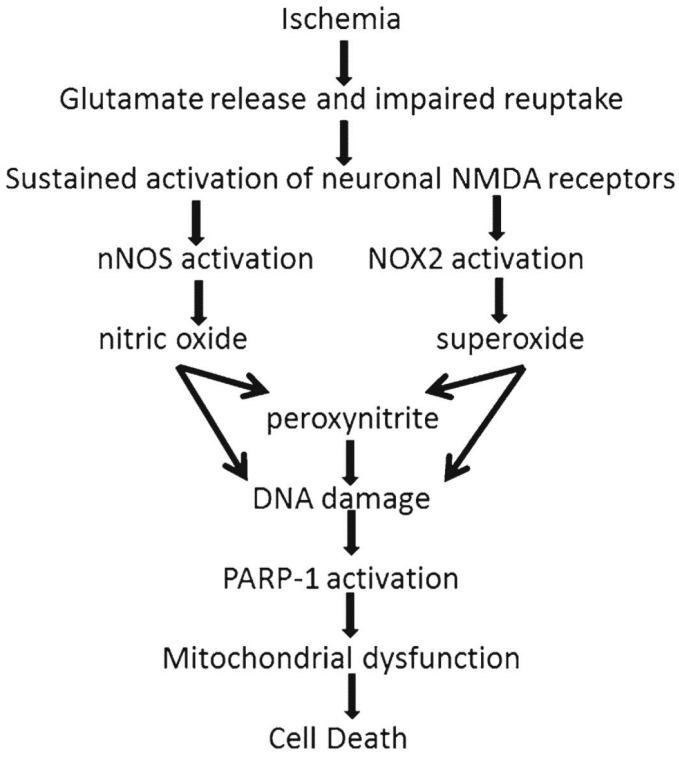

Fig. 1.

PARP-1 and mitochondria in the excitotoxic cell death pathway. Ischemia produces both glutamate release and impaired glutamate reuptake, resulting in sustained action at NMDA-type glutamate receptors. This, in turn, triggers neuronal nitric oxide and superoxide production, by nNOS and NOX2, respectively. Both nitric oxide and superoxide can independently react with DNA, and they can also combine with each other to form the much more reactive compound, peroxynitrite. Extensive DNA damage induces extensive activation of poly(ADP-ribose) polymerase (*PARP-1*), which, in turn, causes mitochondrial depolarization and the onset of mitochondrial cell death programs. For simplicity, the diagram omits many regulatory factors and other contributory factors that influence excitotoxic cell death

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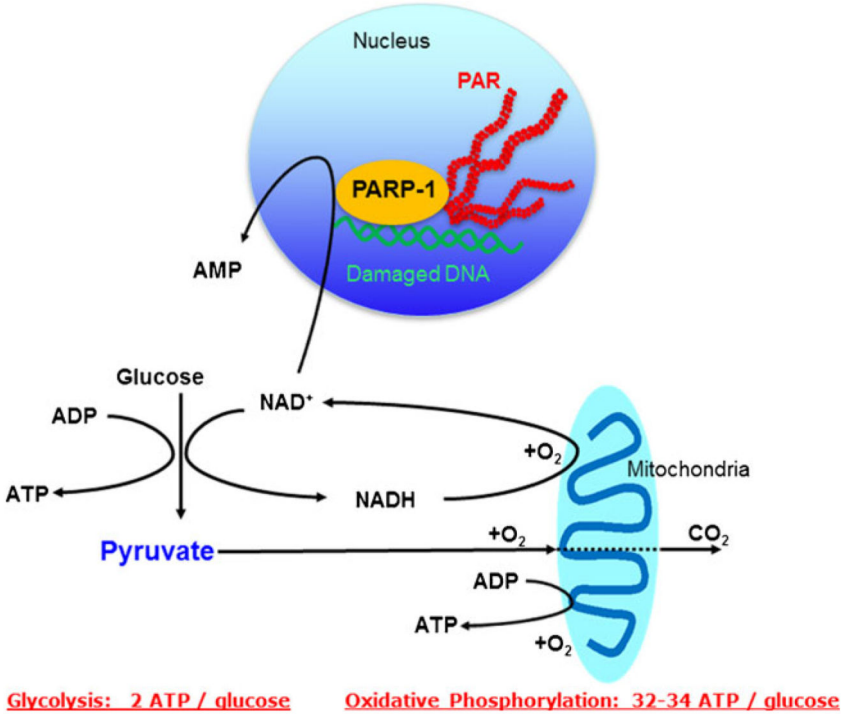

Fig. 2.

Pyruvate can rescue mitochondria when cytosolic NAD^+ is consumed by PARP-1. Glycolysis produces only two ATPs per molecule of glucose consumed, but glycolytic substrates are required for mitochondrial oxidative metabolism, which produced 32-34 additional ATPs per molecule of glucose (depending on the mechanism by which NADH equivalents are transported across the mitochondrial membrane). In the brain, unlike most other tissues, energy metabolism is supported almost entirely by glucose. However, when available, brain mitochondria can also use exogenous pyruvate or other ketone bodies to fuel oxidative metabolism. Mitochondrial utilization of glucose requires the presence of cytosolic NAD^+, but mitochondrial utilization of pyruvate and other ketone bodies does not. In ischemia reperfusion, cytosolic NAD^+ can be consumed by PARP-1 activity in the nucleus. Under these conditions, glucose cannot fuel glycolytic ATP metabolism, and there is, consequently, no carbon flux to mitochondrial for oxidative metabolism, even when oxygen supply is reestablished. The resultant mitochondrial depolarization promotes mitochondrial cell death pathways (parthanatos, apoptosis, energy failure). This can be prevented by supplying exogenous pyruvate or other ketone bodies that are metabolized by mitochondria in the absence of cytosolic NAD^+

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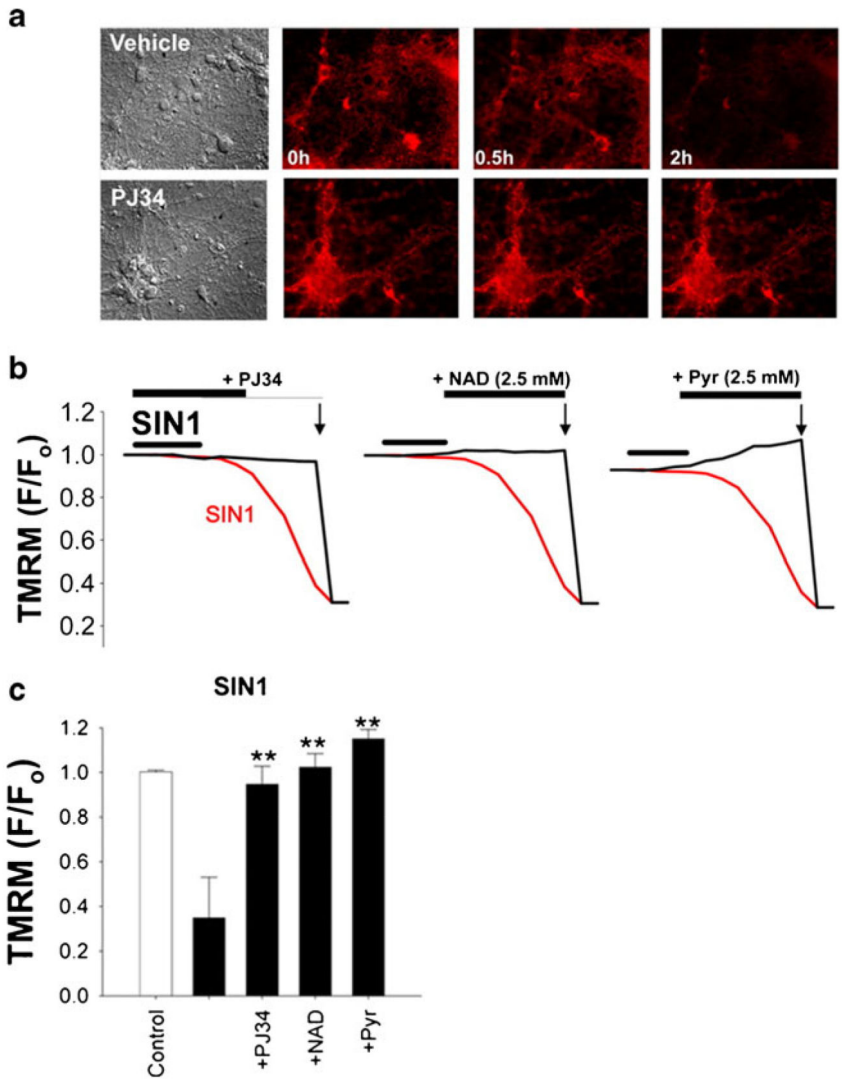

Fig. 3.

PARP-1-induced mitochondrial depolarization is prevented by NAD^+ and pyruvate. **a** Representative images of tetramethylrhodamine (*TMRM*) fluorescence in neurons after 30 min of incubation with 2 mM SIN1, which generates the oxidant peroxynitrite in aqueous solutions. The loss of TMRM fluorescence, indicating loss of mitochondrial membrane potential, was prevented by the PARP-1 inhibitor PJ34 (200 nM). **b** Mitochondrial depolarization over time after PARP-1 activation (*red traces*) by incubation with SIN1 (2 mM). Mitochondrial depolarization was prevented by coincubation with PJ34 (200 nM) or by postincubation with either NAD^+ (2.0 mM) or pyruvate (2.5 mM). Neurons were exposed to FCCP (1 μM) at the end of each experiment to calibrate complete mitochondrial depolarization (*arrows*). **c** Quantified TMRM fluorescence; n =3; $^{**}p$ <0.001 vs. SIN1 alone. Modified from the study of Alano et al. [73]

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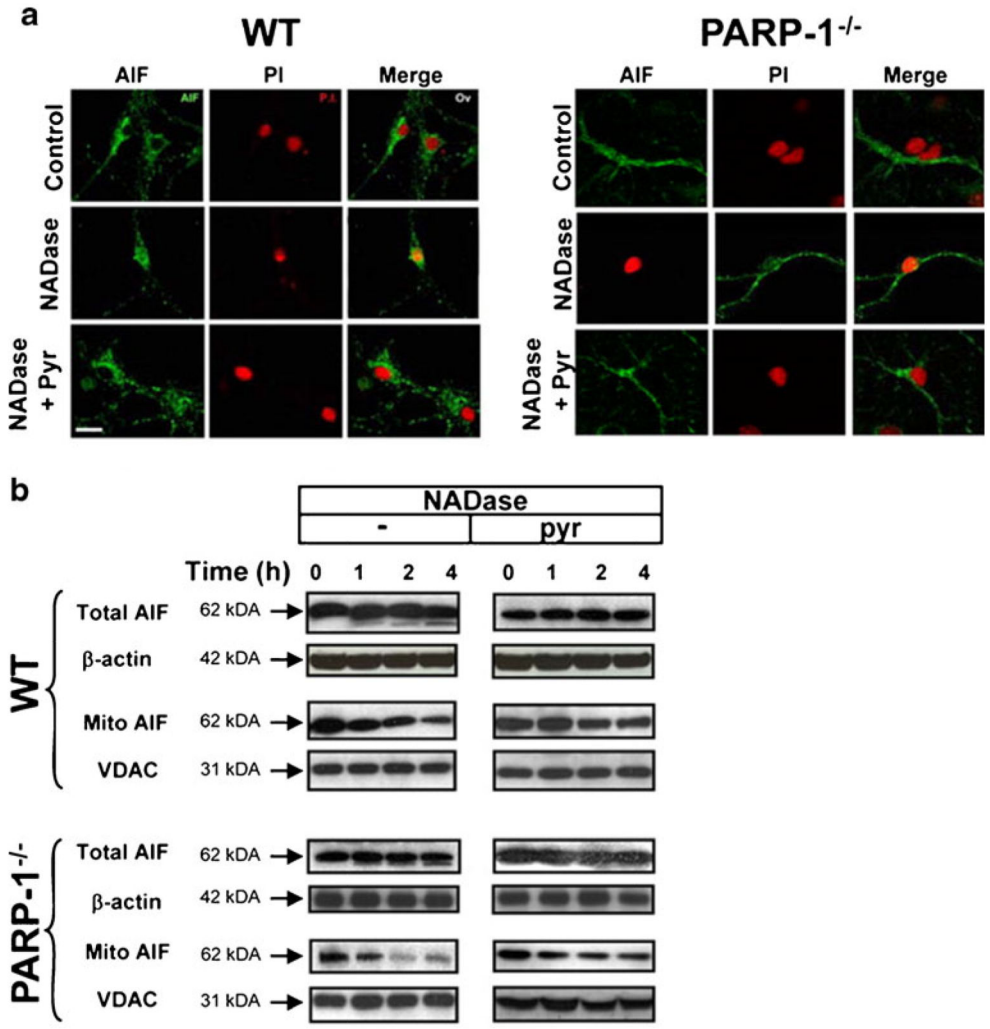

Fig. 4.

AIF translocation induced by PARP-1 or NADase is prevented by pyruvate. **a** Immunostaining for apoptosisinducing factor (*AIF*, *green*) in wild-type or PARP-1$^{-/-}$ neuronal cultures fixed 4 h after transfection with exogenous NADase, which consumes cytosolic NAD^+. Nuclei are counterstained with propidium iodide (*PI*, *red*). Merged images show that pyruvate (2.5 mM) blocks AIF translocation to the nucleus. Images are representative of four independent experiments; *scale bar* =40 μm. **b** Western blots show both total and mitochondrial AIF content at designated time points (in hours) after transfection with NADase, with or without 2.5 mM pyruvate added to the culture medium. The western blots were quantified after normalizing to either β-actin for total AIF or the mitochondrial protein voltage-dependent anion channel (*VDAC*) for mitochondrial AIF. Mitochondrial AIF release was blocked by 2.5 mM pyruvate. Modified from the study of Alano et al. [73]

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Table 1

Protection by pyruvate and other ketone bodies in animal models of ischemia

Paper	Species	Stroke model	Molecule	Dose and timing	Effect
Vergoni et al. [76]	Adult male rats	TGI	γ-Hydroxybutyrate	300 mg/kg i.p. 30 min before or 10 min after insult, with 100 mg/kg injections twice daily for 10 days	Reduction in infarct size and behavioral deficit
Lee et al. [77]	Adult male rats	TGI	Pyruvate	500 mg/kg injection 0.5–1.0 h postischemia	Reduction in infarct size
Suzuki et al. [78]	Adult male rats	PMCAO and TMCAO	β-Hydroxybutyrate	30 mg/kg/h by continuous intra-arterial delivery for 24 to 72 h, beginning immediately after ischemia	Reduction in infarct size, lipid peroxidation, and behavioral deficit; preservation of ATP levels
Ottani et al. [79]	Adult male rats	Intrastriatal injection of endothelin-1	γ-Hydroxybutyrate	100-300 mg/kg i.p. 2 h postischemia, with subsequent 50 or 100 mg/kg injections every 12 h for 10 days	High dose improved motor function and spatial memory and reduced demyelination and necrosis
Gonzalez-Falcon et al. [80]	Adult male rats	PMCAO	Pyruvate	250, 500, and 1,000 mg/kg i.p. 30 min before or after insult	Lower doses reduced behavioral deficits and mortality, but no reduction in infarct volume
Yu et al. [75]	Adult male rats	TMCAO	Ethyl pyruvate	1–40 mg/kg i.p. 30 min preischemia and 4, 12, and 24 h postischemia	Reduced infarct volume and behavioral deficits
Sadasivan et al. [81]	Adult male rats	TMCAO	γ-Hydroxybutyrate, γ-butyrolactone, 1,4-butanediol	300 mg/kg i.p. 30 min before ischemia and 180 and 360 min after	Reduced infarct volume
Yi et al. [82]	Adult male rats	TMCAO, PMCAO	Pyruvate	31.3–500 mg/kg i.p. or i.v. 30 min or 1 or 3 h postinsult	Reduced infarct size and motor deficits in both TMCAO and PMCAO, by both i.v. and i.p. administrations, given up to 1 h after insult
Sharma et al. [83]	Mongrel dogs	Cardiopulmonary arrest for 5 min	Pyruvate	0.125 μmol/kg/min for 1 h	Reduced behavioral deficit, hippocampal neuron loss, caspase-3 activation, DNA fragmentation
Gao et al. [84]	Male mice	TMCAO	γ-Hydroxybutyrate	100 mg/kg i.p. at ischemia and twice daily for 10 days	Accelerated recovery of weight and grip strength, but no reduction in neuronal death or reactive astrocytes
Wang et al. [85]	Male rats	TMCAO for 2 h; 5 mg/kg LPS IP injection	Pyruvate	500 mg/kg i.p. 10 min before reperfusion	Reduced infarct volume, behavioral deficits, microglial activation, and NFkB activation
Martin et al. [86]	Adult male rats	TMCAO	Pyruvate	400 mg/kg i.p. 45 min postischemia	Transiently improved behavioral deficit and ATP levels
Shen et al. [87]	Neonatal rats	Left common carotid artery occlusion with 2.5 h of hypoxia (8 % O_2)	Ethyl pyruvate	50 mg/kg i.p. 30 min pre- or postischemia	Reduced infarct size, behavioral deficit, and microglial activation
Pan et al. [88]	Neonatal rats	Left common carotid artery occlusion with 2.5 h of hypoxia (8 % O_2)	Pyruvate	500 mg/kg i.p. 5 min postischemia	Reduced infarct volume and motor deficits

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Paper	Species	Stroke model	Molecule	Dose and timing	Effect
Xu et al. [89]	Male rats	Cardiac arrest for 7 min, TMCAO	C_4 ketone bodies; dipropionyl cysteine ethyl ester	Ketogenic diet for 3 weeks; DPNCE 450 μmol/kg/min for 30 min after ischemia	Reduced mortality after cardiac arrest; reduced infarct volume after TMCAO
Ryou et al. [90]	Male rats	TMCAO	Pyruvate	50 μmol/kg/min i.v. for 90 min starting 30 min before reperfusion	Reduced infarct volume and DNA fragmentation

Sixteen publications were found in which ketone bodies were used in animal models of stroke. Thirteen of the 16 described reduced cell death or infarct volume. Proposed mechanism of neuroprotection include free radical scavenging, Zn^{2+} /Ca^{2+} chelation, energy substrate supplementation, suppressing inflammation, activating erythropoietin signaling, and, for gamma- and beta-hydroxybutyrate, effects on neuronal activity or sleep

TGI transient global (forebrain) ischemia, *TMCAO* transient middle cerebral artery occlusion, *PMCAO* permanent middle cerebral artery occlusion, *i.p.* intraperitoneal, *i.v.* intravenous