

Low-Dose Poly(ADP-Ribose) Polymerase Inhibitor–Containing Combination Therapies Reverse Early Peripheral Diabetic Neuropathy

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Poly(ADP-ribose) polymerase (PARP) inhibition has recently been identified as a novel approach to treatment of experimental peripheral diabetic neuropathy (PDN). However, long-term inhibition of PARP, an enzyme involved in DNA repair, can potentially result in premature aging, loss of genome stability, and other side effects. This study evaluated potential synergistic interactions between low doses of the potent and specific PARP inhibitor 1,5-isoquinolinediol (ISO) and one of two vasodilators, the ACE inhibitor lisinopril (LIS) and the β_2 -adrenoceptor agonist salbutamol (SAL) in the model of early PDN. Control and streptozotocin (STZ)-induced diabetic rats were treated with either ISO plus LIS or ISO plus SAL for 2 weeks after an initial 2 weeks without treatment. ISO (intraperitoneally) and LIS and SAL (both in the drinking water) were used in subtherapeutic doses, resulting in a minor correction of diabetes-associated sciatic motor and hind-limb digital sensory nerve conduction deficits when administered as monotherapies. Both combination treatments corrected endoneurial blood flow and vascular conductance deficits in STZ-induced diabetic rats. ISO plus SAL corrected all other changes of PDN, i.e., motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) deficits as well as thermal and mechanical hyperalgesia. With ISO plus LIS, no significant correction of MNCV was observed, and the effect on thermal hyperalgesia was quite modest. SNCV and mechanical hyperalgesia were corrected. In vitro studies in human endothelial and Schwann cells showed early accumulation of poly(ADP-ribosyl)ated proteins (Western blot analysis) in response to high glucose, thus suggesting the importance of PARP activation in human PDN. In conclusion, low-dose PARP inhibitor–containing combination therapies may constitute a new approach for treatment of PDN. *Diabetes* 54:1514–1522, 2005

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HSC, human Schwann cell; ISO, 1,5-isoquinolinediol; LIS, lisinopril; MAPK, mitogen-activated protein kinase; MNCV, motor nerve conduction velocity; NBF, nutritive blood flow; PARP, poly(ADP-ribose) polymerase; PDN, peripheral diabetic neuropathy; RNAi, RNA interference; SAL, salbutamol; SNCV, sensory nerve conduction velocity; STZ, streptozotocin; TNF- α , tumor necrosis factor- α .

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Growing evidence indicates that poly(ADP-ribose) polymerase (PARP) activation, a downstream effector of free radical and oxidant-induced DNA single-strand breakage, is an important mechanism in the pathogenesis of diabetes complications (1–4). PARP activation leads to 1) NAD⁺ depletion and energy failure (5); 2) changes in transcriptional regulation and gene expression (5–7); and 3) poly(ADP-ribosyl)ation and resulting inhibition of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, with concomitant activation of several major pathogenetic mechanisms, i.e., nonenzymatic glycation, protein kinase C, and hexosamine pathway (2). Using a pharmacological approach with several structurally unrelated PARP inhibitors as well as PARP-deficient mice, we have recently discovered the key role of PARP in the development of early peripheral diabetic neuropathy (PDN) (3,8). Note that PARP inhibitors administered in doses resulting in complete or almost complete PARP inhibition in the diabetic peripheral nerve (3,8) appeared at least 100-fold more effective in correcting nerve conduction slowing, NAD⁺/NADH redox imbalances, energy failure, and neurovascular dysfunction than conventional antioxidants (9,10).

A recent 9-month PARP inhibitor study in the streptozotocin (STZ)-induced diabetic rat model did not reveal clearly manifest side effects (4), and PARP^{-/-} mice do not develop obvious phenotypic changes (5). However, the long-term consequences of complete PARP inhibition in pathological conditions associated with oxidative stress, including diabetes, are unclear and can hardly be sorted out in rodents because of their limited life span. Diabetes-associated oxidative stress leads to DNA damage, e.g., in the peripheral nervous system (11), and PARP activation is an important mechanism of DNA repair (5). Therefore, it is reasonable to suggest that 1) complete PARP inhibition can potentially result in premature aging and 2) targeted delivery of PARP-1 antisense oligodeoxynucleotide-containing vectors (12) or PARP-1 RNA interference (RNAi) (13), rather than pharmacological PARP inhibition, should be considered as a future therapy of PDN (1). However, it is unlikely that such approaches will be available in clinical practice in the near future. An alternative strategy would be to develop low-dose PARP inhibitor–containing combination therapies with other agents already used in clinical practice and proven to be effective in the treatment

of early experimental PDN. Here, we describe synergistic interactions between the PARP inhibitor 1,5-isoquinolinediol (ISO) and two other agents, the ACE inhibitor lisinopril (LIS) and the β_2 -adrenoceptor agonist salbutamol (SAL), in reversal of neuropathic changes in the STZ-induced diabetic rat model. We also provide the first evidence of early high-glucose-induced PARP activation in human endothelial and Schwann cells, suggesting the importance of PARP activation in human PDN (M. Brownlee, discussion at the NIH conference "Diabetic Complications: Progress through Animal Models, 20–21 October 2003, Bethesda, MD, unpublished).

RESEARCH DESIGN AND METHODS

The experiments were performed in accordance with regulations specified by the National Institutes of Health Principles of Laboratory Animal Care, 1985 Revised Version and University of Michigan Protocol for Animal Studies.

Reagents. Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma Chemical Co. (St. Louis, MO). Human endothelial cells (aortic, HAAE-1) and growth medium were purchased from American *Type Culture* Collection (Manassas, VA), and human Schwann cells (HSCs) and growth medium were purchased from ScienCell Research Laboratories (San Diego, CA). PARP-1 antibody (clones C2–10) and monoclonal poly(ADP-ribose) antibody were purchased from Biomol International (Plymouth Meeting, PA), and the anti-mouse horseradish peroxidase-conjugated secondary antibody was purchased from Cell Signaling (Beverly, MA).

Animal model. Male Wistar rats (Charles River, Wilmington, MA), 250- to 300-g body weight, were fed a standard rat diet (PMI Nutrition Int., Brentwood, MO) and had access to water ad libitum. Diabetes was induced by STZ as we described previously (3,8). Blood samples for glucose measurements were taken from the tail vein ~48 h after the STZ injection and the day before the animals were killed. The rats with blood glucose ≥ 13.8 mmol/l were considered diabetic.

Design of PARP inhibitor-containing combination therapies. In our previous studies (3,8), three structurally diverse PARP inhibitors, i.e., 3-aminobenzamide, ISO, and PJ34, corrected nerve conduction deficits and energy deficiency, but only two of them, 3-aminobenzamide and ISO, essentially corrected neurovascular dysfunction, i.e., endoneurial nutritive blood flow (NBF) and vascular conductance in STZ-induced diabetic rats. From these observations, we concluded that PARP inhibition counteracts diabetes-induced changes in nerve energy state (the variable that correlates best with nerve conduction) primarily via a recently discovered effect on the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (2) and resulting improvement of glucose utilization in Schwann cells. The latter is consistent with normalization of free NAD^+ -to-NADH ratio, an index of tricarboxylic cycle activity and glucose utilization, in the peripheral nerve mitochondrial matrix of PJ34-treated diabetic rats that was achieved despite relatively modest (17%) correction of NBF (8). Of the three PARP inhibitors (3,8), we have selected the potent and specific ISO, which, at the very low dose of $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ i.p., almost completely blunted nerve poly(ADP-ribose) accumulation (a marker of PARP activation) and reversed early PDN in STZ-induced diabetic rats. Two other agents used in the present study, the ACE inhibitor LIS and the β_2 -adrenoceptor agonist SAL, are typical, very potent vasodilators that have been reported to reverse nerve conduction deficits via correction of nerve blood flow and endoneurial hypoxia at the doses of 10 and $0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, respectively, in the same animal model (14,15). Furthermore, LIS improved nerve conduction and vibration perception thresholds in human PDN (16). Thus, the design of the ISO plus LIS and ISO plus SAL combination therapies was based on the premise that two classes of agents, i.e., PARP inhibitors and vasodilators, counteract diabetes-induced peripheral nerve energy deficiency and resulting dysfunction via two different but complementary mechanisms: 1) disinhibition of the second half of glycolysis and glucose utilization in the tricarboxylic acid cycle, and 2) restoration of normal nerve oxygenation and correction of oxidation-phosphorylation uncoupling.

In our preliminary combination therapy dose-finding study, the monotherapies with LIS ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) or SAL ($0.05 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$), both in the drinking water, for 2 weeks after 4 weeks without treatment resulted in a slight (~15–25%) correction of final motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) deficits (MNCV: controls 57.56 ± 0.82 m/s; diabetic 48.02 ± 0.765 m/s; diabetic plus LIS 49.24 ± 0.47 m/s; diabetic plus SAL 49.73 ± 0.82 m/s; SNCV: controls 39.23 ± 1.02 m/s; diabetic 32.87 ± 1.11 m/s; diabetic plus LIS 34.4 ± 0.68 m/s; diabetic plus SAL 34.52 ± 1.17 m/s; means \pm SE, $n = 4$) The ISO monotherapy, $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ i.p.,

caused ~50% correction of SNCV deficit without any effect on MNCV deficit (SNCV: 36.09 ± 1.37 m/s; MNCV: 48.20 ± 0.75 m/s; $n = 4$; the corresponding values for controls and the untreated diabetic group are provided above). These subtherapeutic doses of three agents have been selected for the present study, in which the experimental groups comprised control and diabetic rats treated with or without one of the two combination therapies, i.e., ISO plus LIS or ISO plus SAL. The treatments were started 2 weeks after the initial 2 weeks without treatment. The duration of the experiment was 4 weeks.

Anesthesia, euthanasia, and nerve sampling. Rats were anesthetized by inactin ($65\text{--}85 \text{ mg/kg}$ body wt i.p.). The onset (before induction of diabetes) MNCV and SNCV measurements were followed by the 2-week time point (before interventions) and final (4-week time point) measurements. In all measurements, body temperature was monitored by a rectal probe and maintained at 37°C with a warming pad. Hind-limb skin temperature was also monitored by a thermistor and maintained between 36 and 38°C by radiant heat. After completion of nerve functional measurements, the animals were sedated by CO_2 in a specially designed chamber (10) and immediately killed by cervical dislocation. The left nerves were rapidly dissected, blotted with fine filter paper to remove any accompanying blood, and frozen in liquid nitrogen for subsequent preparation of lysates and Western blot analysis of PARP-1 and poly(ADP-ribose).

HAAE-1 and HSC cell cultures. Human endothelial and Schwann cells were cultured in commercial media, according to manufacturer's instructions. Passages 7–10 were used in all experiments.

Functional studies

Sciatic MNCV, digital SNCV, and sciatic endoneurial NBF and vascular conductance. These variables have been measured as described previously (3,10).

Thermal algia. To determine the sensitivity to noxious heat, rats were placed within a Plexiglas chamber on a transparent glass surface and allowed to acclimate for at least 20 min. A thermal stimulation meter (IITC Life Science Instruments, Woodland Hills, CA) was used. The device was activated after placing the stimulator directly beneath the plantar surface of the hind paw. The paw withdrawal latency in response to the radiant heat (17% intensity) was recorded. Individual measurements were repeated four to five times, and the mean value was calculated as the thermal threshold.

Mechanical algia. Sensitivity to noxious mechanical stimuli was determined by quantifying the withdrawal threshold of the hind paw in response to mechanical stimulation using a von Frey Anesthesiometer (model 2290C; IITC Life Science). The test was conducted between 9:00 and 11:30 A.M. each day. The rats were placed in individual Plexiglas boxes on a stainless-steel mesh floor and were allowed to acclimate for at least 20 min. A 0.5-mm diameter polypropylene rigid tip was used to apply a force to the plantar surface of the hind paw. The force causing the withdrawal response was recorded by the anesthesiometer. The anesthesiometer was calibrated before each recording. The test was repeated four to five times at ~5-min intervals on each animal, and the mean value was calculated.

Western blot analysis of PARP and poly(ADP-ribose): sciatic nerves. Frozen sciatic nerves were weighed, transferred to an extraction buffer (1:5 wt/vol) containing 50 mmol/l Tris-HCl, pH 7.2, 150 mmol/l NaCl, 0.1% SDS, 1% Nonidet P-40, 5 mmol/l EDTA, 1 mmol/l EGTA, 1% sodium deoxycholate, and the protease/phosphatase inhibitors leupeptin (10 $\mu\text{g/ml}$), aprotinin (20 $\mu\text{g/ml}$), benzamide (10 mmol/l), phenylmethylsulfonyl fluoride (1 mmol/l), and sodium orthovanadate (1 mmol/l) and homogenized on ice. The homogenate was sonicated (3×5 s) and centrifuged at 14,000g for 20 min. Tissue lysates were precleared by constant mixing with protein G-Sepharose for 1 h. All of the aforementioned steps were performed at 4°C . The lysates (100 μg protein) were mixed with equal volume of $2\times$ sample loading buffer containing 62.5 mmol/l Tris-HCl, pH 6.8, 2% SDS, 5% β -mercaptoethanol, 10% glycerol, and 0.025% bromophenol blue and fractionated in 5–17% SDS-PAGE in an electrophoresis cell (Mini-Protean III; Bio-Rad Laboratories, Richmond, CA). Electrophoresis was conducted at 15 mA constant current for stacking and at 25 mA for protein separation. Gel contents were electrotransferred (250 mA, 2 h) to nitrocellulose membranes using Mini Trans-Blot cell (Bio-Rad Laboratories) and Western transfer buffer (25 mmol/l Tris-HCl, pH 8.3, 192 mmol/l glycine, and 20% [vol/vol] methanol) (17). Free binding sites were blocked in 5% (wt/vol) nonfat dry milk in 20 mmol/l Tris-HCl buffer, pH 7.5, containing 150 mmol/l NaCl and 0.05% Tween 20 for 1 h, after which PARP-1 or poly(ADP-ribose) antibodies were applied for 2 h for detection of PARP-1 and poly(ADP-ribose)ated proteins, respectively. The horseradish peroxidase-conjugated secondary antibody was then applied for 1 h. Negative controls for each sample contained no primary antibody. After extensive washing, protein bands detected by the antibodies were visualized with the BM Chemiluminescence Blotting Substrate (POD; Roche, Indianapolis, IN). Total content of all sciatic nerve poly(ADP-ribose)ated proteins was quantified by densitometry (Quantity One 4.5.0 software; Bio-Rad Laboratories). The peak \times area values

TABLE 1

Body weights and final blood glucose concentrations in control and diabetic rats treated with or without ISO plus LIS and ISO plus SAL combinations

	Body weight (g)		Blood glucose (mmol/l)
	Initial*	Final	
C	275 ± 8	432 ± 9	4.83 ± 0.20
C + ISO + LIS	281 ± 7	420 ± 21	5.07 ± 0.19
C + ISO + SAL	270 ± 9	427 ± 6	6.03 ± 0.44
D	284 ± 8	361 ± 9†	26.3 ± 1.17†
D + ISO + LIS	273 ± 6	347 ± 11†	25.1 ± 2.03†
D + ISO + SAL	276 ± 8	370 ± 12†	27.4 ± 1.50†

Data are means ± SE (n = 10–15). *Before induction of STZ-induced diabetes; †significantly different from controls (P < 0.01). C, control rats; D, diabetic rats.

were corrected for the corresponding negative controls. Membranes were then stripped in the 62.5 mmol/l Tris-HCl, pH 6.7, buffer containing 2% SDS and 100 mmol/l β-mercaptoethanol and reprobed with β-actin antibody to confirm equal protein loading.

Western blot analysis of PARP and poly(ADP-ribose): HAAE-1 and HSCs. To assess PARP-1 and poly(ADP-ribose) by Western blot analysis, HAAE-1 and HSCs were cultured for 12 h in commercial media containing 1) 7 mmol/l (endothelial cells) or 5.5 mmol/l (Schwann cells) glucose, 2) 30 mmol/l glucose, and 3) 30 mmol/l glucose plus 20 μmol/l ISO. Cells were lysed in the 2× sample buffer containing 62.5 mmol/l Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.025% bromphenol blue. Western blot analyses were performed as described above.

Statistical analysis. The results are expressed as means ± SE. Data were subjected to an equality of variance F test and then to log transformation, if necessary, before one-way ANOVA. Where a overall significance (P < 0.05) was attained, individual between-group comparisons were made using the Student-Newman-Keuls multiple range test. Significance was defined at P < 0.05. When between-group variance differences could not be normalized by log transformation (datasets for body weights, plasma glucose, and some metabolic parameters), the data were analyzed by the nonparametric Kruskal-Wallis one-way ANOVA, followed by the Bonferroni/Dunn test for multiple comparisons.

RESULTS

The final body weights were lower in untreated diabetic rats and diabetic rats receiving combination therapies than in the control group (Table 1). ISO plus LIS and ISO plus SAL treatments did not affect weight gain in either nondiabetic or diabetic rats. The final blood glucose concentrations were similarly elevated in the untreated and low-dose PARP inhibitor-treated diabetic rats compared with the control rats. The onset (before induction of STZ-induced diabetes) and 2-week time point (before interven-

TABLE 2

The onset* and 2-week MNCVs and SNCVs in control and diabetic rats treated with or without ISO plus LIS and ISO plus SAL combinations

	MNCV		SNCV	
	Onset	2-week	Onset	2-week
C	55.5 ± 1.2	56.5 ± 1.0	36.7 ± 0.5	37.6 ± 0.4
C + ISO + LIS	55.7 ± 0.7	56.7 ± 0.9	37.0 ± 0.6	38.1 ± 0.7
C + ISO + SAL	55.2 ± 0.9	57.0 ± 1.1	36.5 ± 0.5	37.7 ± 0.6
D	55.6 ± 0.6	45.9 ± 1.0†	36.8 ± 0.6	32.3 ± 0.4†
D + ISO + LIS	56.0 ± 0.8	45.6 ± 1.1†	36.4 ± 0.6	31.9 ± 0.5†
D + ISO + SAL	55.4 ± 0.7	46.3 ± 0.9†	36.9 ± 0.6	32.0 ± 0.4†

Data are means ± SE (n = 10–15). *Before induction of STZ diabetes; †significantly different from the corresponding onset values (P < 0.01). C, control rats; D, diabetic rats.

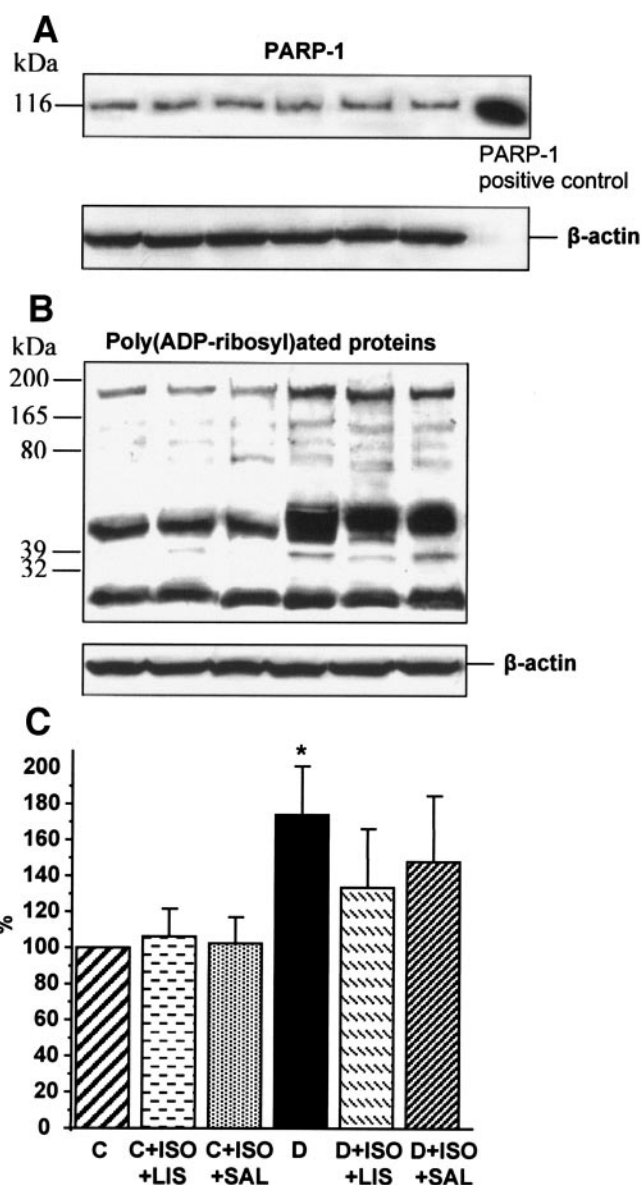


FIG. 1. A and B: Representative Western blot analyses of rat sciatic nerve PARP-1 and poly(ADP-ribosyl)ated proteins, respectively. Equal protein loading was confirmed with β-actin antibody. C: Rat sciatic nerve total poly(ADP-ribosyl)ated protein content in control (C) and diabetic (D) rats treated with or without ISO plus LIS or ISO plus SAL. Total poly(ADP-ribosyl)ated protein content in control rats is taken as 100%. The results are expressed as means ± SE (n = 6–12 animals/group). *P < 0.05 vs. control group.

tions) MNCV and SNCV were similar among the groups (Table 2).

Sciatic nerve PARP-1 protein expression was similar in control and diabetic rats treated with or without ISO plus LIS or ISO plus SAL (Fig. 1A). Total sciatic nerve poly(ADP-ribosyl)ated protein content (Fig. 1B and C) was increased by 74% in the diabetic rats compared with control rats (P < 0.05). This increase was partially corrected by both combination therapies (to 134 and 148% of the control value, by ISO plus LIS and ISO plus SAL, respectively), but the differences with the untreated diabetic group did not achieve statistical significance. Neither ISO plus LIS nor ISO plus SAL treatments affected total poly(ADP-ribosyl)ated protein content in nondiabetic rats.

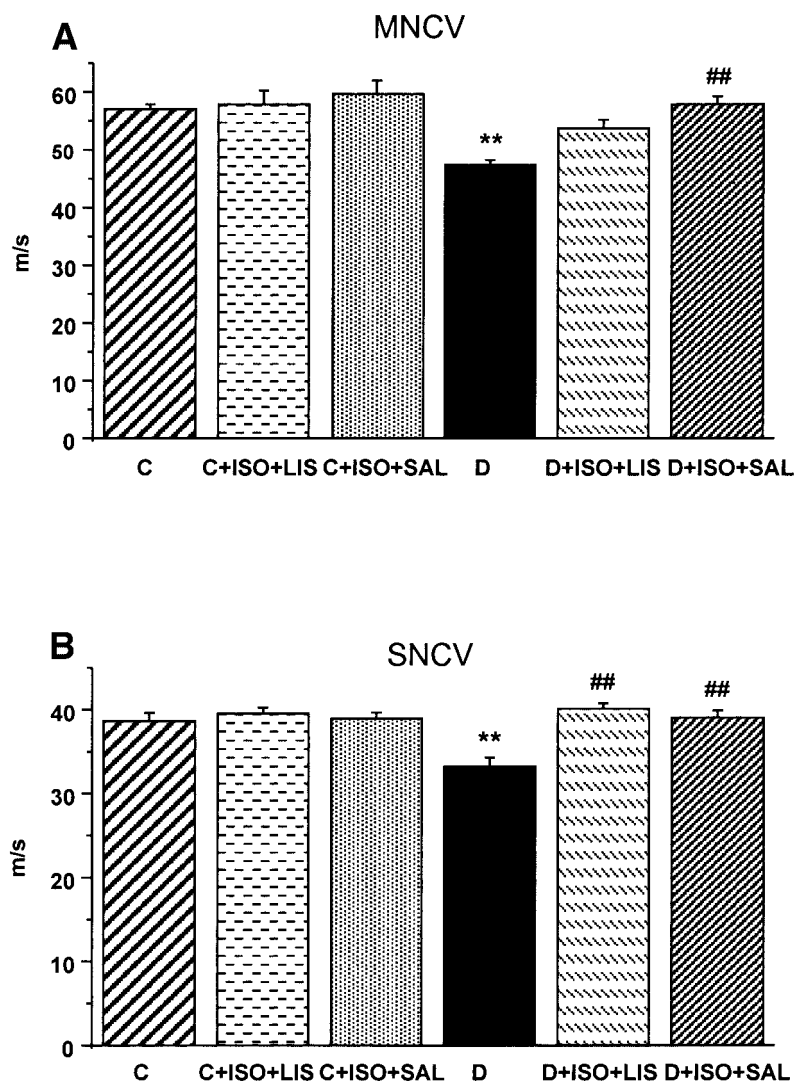


FIG. 2. Final sciatic MNCVs (A) and hind-limb digital SNCVs (B) in control (C) and diabetic (D) rats treated with or without ISO plus LIS or ISO plus SAL. The results are expressed as means \pm SE ($n = 10-15$ animals/group). ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. untreated diabetic group.

The final MNCVs and SNCVs were reduced by 17 and 14% in diabetic rats compared with control rats (Fig. 2). ISO plus LIS therapy normalized SNCVs in diabetic rats. MNCVs tended to increase, but the difference with the untreated diabetic group did not achieve statistical significance ($P > 0.05$). ISO plus SAL treatment normalized both MNCVs and SNCVs in diabetic rats. Neither ISO plus LIS nor ISO plus SAL combination therapies affected MNCVs or SNCVs in control rats.

NBF was reduced by 51% in diabetic rats compared with the control group (Fig. 3A), and this deficit was corrected by ISO plus LIS and ISO plus SAL combination therapies to a similar extent (to 94 and 92% of control value, respectively). Mean systemic blood pressure (Fig. 3B) was reduced by 21% in diabetic rats ($P < 0.01$) and was increased by both combination therapies to the levels that did not significantly differ from those in either control or untreated diabetic rats ($P > 0.05$). Endoneurial vascular conductance (Fig. 3C) was reduced by 42% in diabetic rats compared with control rats, and this deficit was corrected (to 102% of control value) by both combination therapies.

The thermal response latency was decreased by 46% in diabetic rats compared with control rats (Fig. 4A), which is consistent with the development of transient thermal hyperalgesia (18). This variable was partially (to 79% of

control value) corrected by ISO plus LIS treatment ($P < 0.01$ vs. untreated diabetic group, and $P < 0.05$ vs. controls). The thermal response latency was corrected to 88% of the control value by ISO plus SAL treatment ($P < 0.01$ vs. untreated diabetic group). Of interest, both ISO plus LIS and ISO plus SAL therapies slightly reduced paw withdrawal latency in response to noxious stimuli in control rats, and the difference between untreated and ISO plus SAL-treated nondiabetic groups achieved statistical significance ($P < 0.01$).

Diabetic rats developed mechanical hyperalgesia manifest in 65% reduction of paw withdrawal thresholds ($P < 0.01$) (Fig. 5) in response to mechanical noxious stimuli (Fig. 4B). This variable was corrected by both ISO plus LIS and ISO plus SAL therapies (to 100 and 106% of control value, respectively; $P < 0.01$ vs. untreated diabetic group for both comparisons). Neither ISO plus LIS nor ISO plus SAL therapy affected paw withdrawal thresholds in control rats.

PARP-1 protein abundance was not affected by hyperglycemia or the presence of the PARP inhibitor ISO in either human endothelial cells (Fig. 5A) or HSCs (Fig. 5B). Poly(ADP-ribosyl)ated protein abundance was increased in both endothelial and Schwann cells cultured in hyperglycemic versus normoglycemic conditions (Fig. 6A and

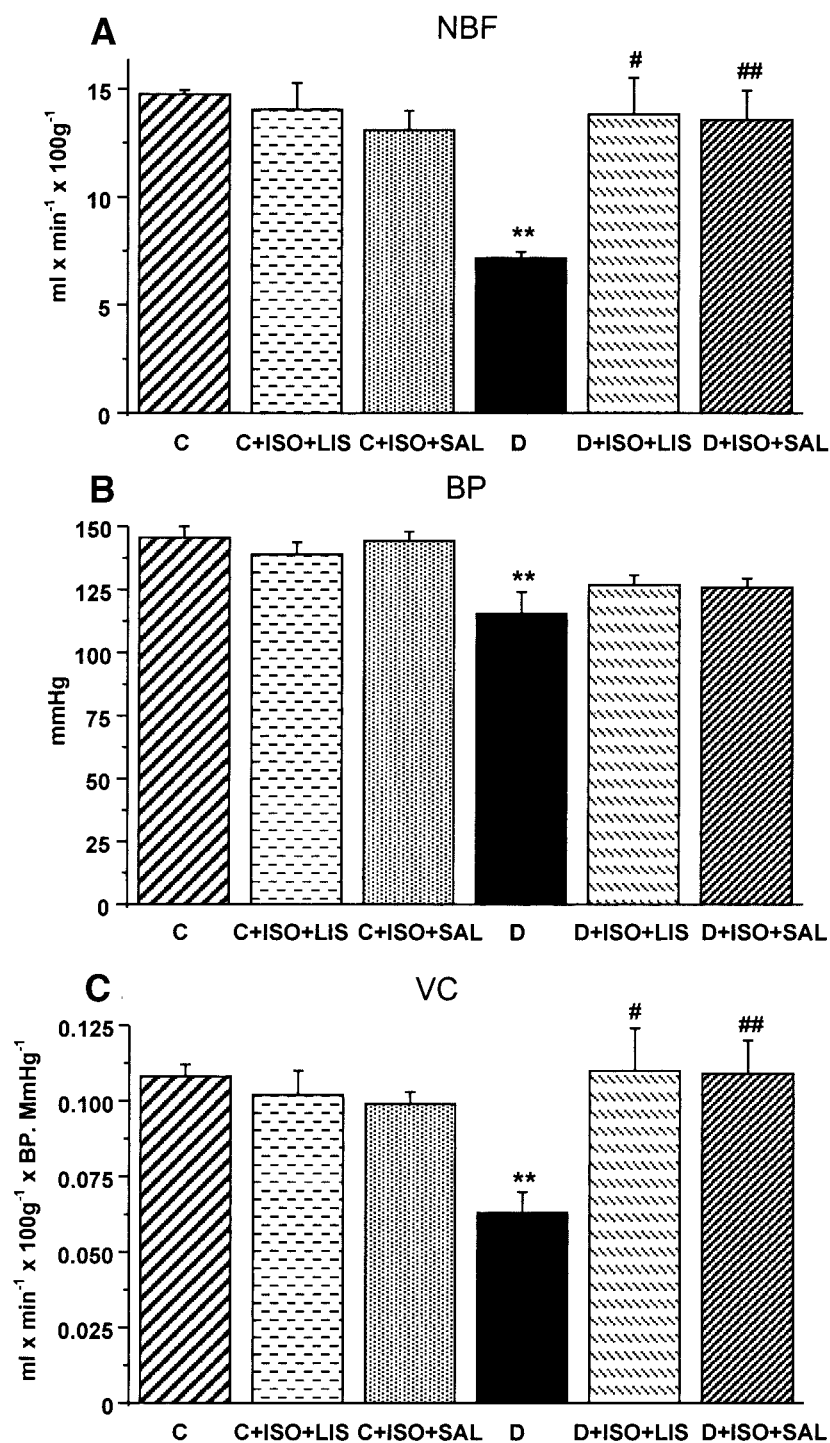


FIG. 3. Sciatic endoneurial blood flow (NBF) (A), mean systemic blood pressure (BP; B), and endoneurial vascular conductance (VC) (C) in control (C) and diabetic (D) rats treated with or without ISO plus LIS or ISO plus SAL. The results are expressed as means \pm SE ($n = 10-15$ animals/group). * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. untreated diabetic group.

B). Hyperglycemia-induced excessive poly(ADP-ribose)ylation was suppressed by ISO in both cell types.

DISCUSSION

A variety of mechanisms have been implicated in the pathogenesis of PDN, including increased aldose reductase activity (19-21), nonenzymatic glycation/glycoxidation (18,22,23), activation of protein kinase C (24), impaired neurotrophic support (25,26), and enhanced oxidative-nitrosative stress (9-13,27) and, recently, downstream effectors of free radical and oxidant-induced injury, i.e., mitogen-activated protein kinase (MAPK) activation

(28), PARP activation (3,8), and impaired calcium signaling (29). All of these mechanisms have been demonstrated to contribute to early PDN and to cause motor and sensory nerve conduction deficits, neurovascular dysfunction, altered sensation, and diabetic neuropathic pain.

To date, any monotherapy of clinical PDN with relatively high doses of therapeutic agents has been a failure (30), both because of low efficacy and adverse side effects, thus emphasizing the need for lower dose combination approaches. Several years ago, it was established that a cross talk between some pathogenetic factors potentiates their cumulative detrimental effect, and thus low-dose

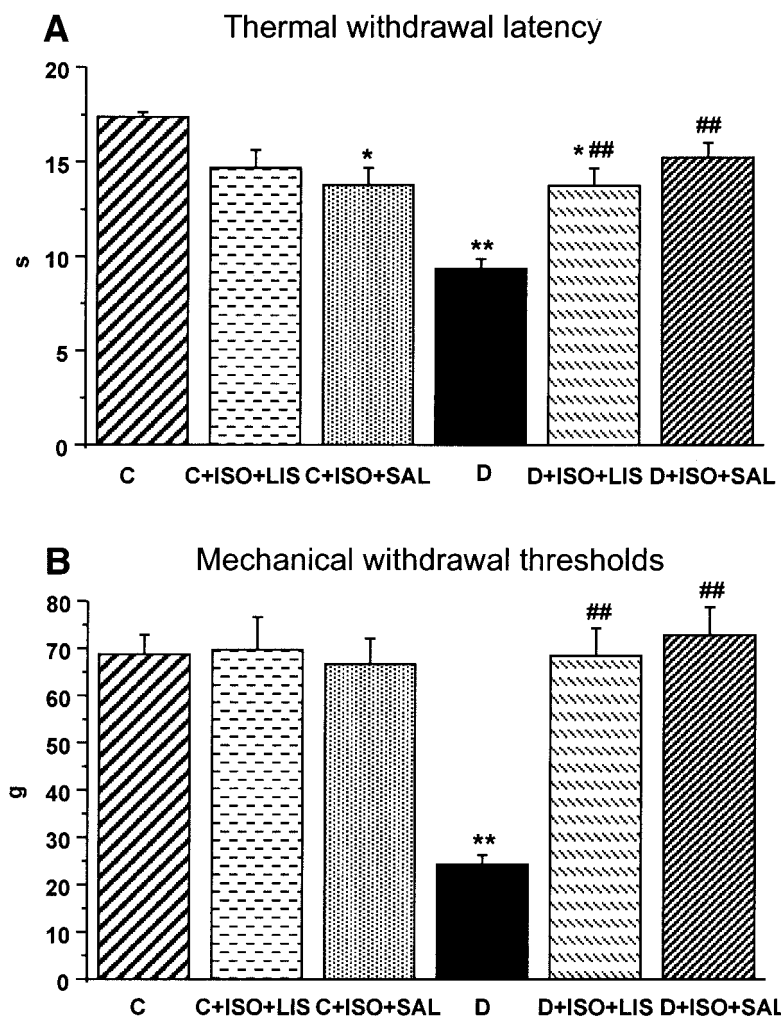


FIG. 4. A: Paw withdrawal latencies in response to thermal noxious stimuli in control (C) and diabetic (D) rats treated with or without ISO plus LIS or ISO plus SAL. The results are expressed as means \pm SE ($n = 6$ animals/group). B: Paw withdrawal thresholds in response to mechanical noxious stimuli in control and diabetic rats treated with or without ISO plus LIS or ISO plus SAL. The results are expressed as means \pm SE ($n = 10$ – 15 animals/group). * $P < 0.05$, ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. untreated diabetic group.

combination therapies containing two or more components are more effective in counteracting early PDN than one would expect from the corresponding monotherapy studies. Synergistic (supra-additive) interactions have been identified between α -lipoic and γ -linolenic acids (31), protein kinase C inhibitor and antioxidants or γ -linolenic acid (32), endothelin 1 ETA and angiotensin I antagonists (33), aldose reductase and ACE inhibitors (14), and aldose reductase inhibitor and α -lipoic acid (34). Almost all ingredients of the combination therapies tested so far have direct or indirect antioxidant properties, consistent with the role of oxidative stress as a convergence point for hyperglycemia-driven pathogenetic pathways (35,36).

Early neuropathic changes can be reversed not only by antioxidants but also by agents counteracting downstream effectors of oxidative stress, e.g., PARP activation (3,8). PARP inhibition alleviates numerous experimental pathologic conditions associated with oxidative stress (5). In diabetes, PARP activation manifest by accumulation of poly(ADP-ribosyl)ated proteins has been described for aortic (1), myocardial (5), and skin microcirculatory (37) endothelial cells; retinal neurons, ganglion cells, and vasculature (4,38); peripheral nerve endothelial and Schwann cells (3) and dorsal root ganglion neurons (39); and tubular and glomerular cells of the renal cortex (7). Correspondingly, complete or almost complete PARP inhibition has been reported to counteract endothelial and

myocardial dysfunction (1,5), PDN (3,8), and retinopathy (4,38). In the present study, both ISO plus LIS and ISO plus SAL therapies, which resulted in partial inhibition of peripheral nerve PARP activation, appeared remarkably effective against multiple manifestations of early PDN, including those that are not amenable to many other treatments (thermal and, especially, mechanical hyperalgesia). Both combination therapies essentially corrected NBF and slightly supranormalized endoneurial vascular conductance. Despite a similar effect on neurovascular dysfunction, only ISO plus SAL therapy normalized both MNCV and SNCV deficits, whereas ISO plus LIS therapy normalized SNCVs only. Note that both LIS and SAL have nonvascular effects. LIS acts as a weak antioxidant and nitric oxide scavenger (40). However, it is unlikely that these effects are manifest with the dose of 0.3 mg/kg used in this study. The spectrum of pharmacological effects of SAL is even more impressive: the agent inhibits expression of intercellular adhesion molecule-1, CD-40, and CD-14 as well as eicosanoid biosynthesis; increases intracellular cAMP concentration, cAMP-dependent protein kinase A, adenylyl cyclase, phosphatase PP2A, and L-type Ca^{2+} channel activities; modulates G-protein signaling; and stimulates pentose phosphate pathway (41–44). At least several of these effects may account for a better MNCV response to ISO plus SAL compared with ISO plus LIS treatment. For example, activation of the pentose phosphate

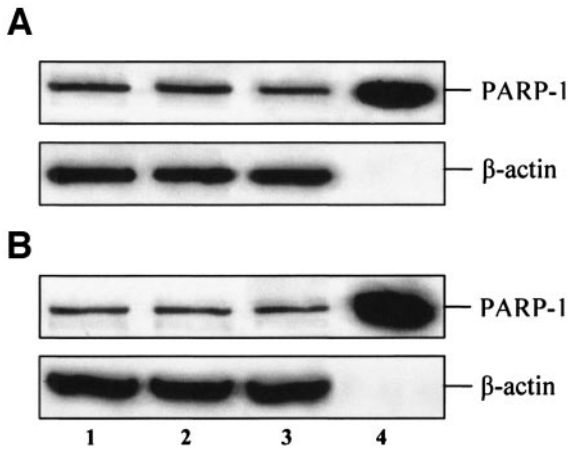


FIG. 5. PARP-1 abundance in human endothelial (A) and HSCs (B). Lane 1: cultured in normoglycemic conditions (7 mmol/l glucose for endothelial cells and 5.5 mmol/l glucose for Schwann cells); lane 2: cultured in hyperglycemic conditions (30 mmol/l glucose); lane 3: cultured in 30 mmol/l glucose plus 20 μmol/l ISO; lane 4: PARP-1 protein (positive control).

pathway can be complementary to PARP inhibitor-caused disinhibition of glycolysis, in stimulating intermediary metabolism of glucose. Inhibition of eicosanoid biosynthesis can eliminate eicosanoid-mediated MAPK kinase and nuclear factor-κB activation and derangements in Ca²⁺ homeostasis. The effects of both LIS and SAL on nonvascular cells and, in particular, Schwann cells of the peripheral nerve require further studies.

Despite considerable knowledge in understanding the pathogenetic mechanisms underlying MNCV and SNCV slowing, key biochemical imbalances, impaired neurotrophic support, and morphological abnormalities characteristic for PDN, the mechanisms of diabetic neuropathic pain and abnormal sensory responses remain remarkably understudied (45). Transient thermal hyperalgesia in rats with short-term STZ-induced diabetes was found to be amenable to prevention/reversal by aldose reductase inhibitors (18), the hydroxymethylglutaryl-CoA reductase inhibitor rosuvastatin (46), the protein kinase C β inhibitor LY333531 (47), α-lipoic acid (48), the hydroxyl radical scavenger dimethylthiourea (49), and taurine (29). Thermal hypoalgesia developing at a later stage of STZ-induced diabetic rats (18) as well as in NOD diabetic mice (27) is prevented by insulin therapy that results in protracted normoglycemia (45), the peroxynitrite decomposition catalyst FP15 (27), aldose reductase inhibitors (18), Schwann

cell-derived ciliary neurotrophic factor (18), and the neurotrophic peptide deriving from prosaposin TX14(A) (45) and is partially prevented by a sonic hedgehog-IgG fusion protein (50). In the present study, thermal hyperalgesia in STZ-induced diabetic rats with a 4-week duration of diabetes was essentially prevented by ISO plus SAL and partially prevented by ISO plus LIS treatment. Mechanical hyperalgesia (an exaggerated response to noxious mechanical stimulation by rigid von Frey filaments) known to be amenable to prevention/reversal by antioxidants (29,48,49) but not several other pharmacological agents (46,47) was completely corrected by both ISO plus LIS and ISO plus SAL combination therapies. PARP activation can contribute to diabetic neuropathic pain via several mechanisms, i.e., 1) upregulation of tumor necrosis factor-α (TNF-α) and other inflammatory genes, 2) activation of p38 MAPK kinase in the spinal cord and Schwann cells, and 3) Ca²⁺-regulated excitotoxic insults, all of which have been implicated in the pathogenesis of painful neuropathy. PARP dependence of TNF-α overexpression, p38 MAPK kinase activation, and Ca²⁺-regulated excitotoxic insults in pathological conditions associated with oxidative stress has been experimentally documented (5).

A recent clinical study revealed an increased percentage of PARP-positive endothelial nuclei in forearm skin microcirculation in subjects at risk of developing type 2 diabetes and those with overt type 2 diabetes (37). Using Western blot analysis, we found accumulation of poly(ADP-ribosyl)ated proteins (an index of PARP activation) to develop very early, i.e., within ~12 h of exposure of both human endothelial and HSCs to hyperglycemic conditions. This is consistent with the study in bovine aortic endothelial cells (2) in which PARP activity was assessed by a different method, i.e., [³H]NAD⁺ radiolabel incorporation into protein. PARP-1 protein abundance was not affected by hyperglycemia or ISO treatment in either cell type consistent with the current knowledge on PARP-1 as abundantly expressed in most cell types' enzyme with very minor, if any, transcriptional regulation (5).

In conclusion, increased poly(ADP-ribosyl)ation is an early response of both human endothelial and Schwann cells to high glucose, which suggests the importance of PARP activation in the pathogenesis of human PDN. Low-dose PARP inhibitor-containing combination therapies with two agents currently used in the clinical practice, i.e., the ACE inhibitor LIS or β₂-adrenoceptor agonist SAL, are remarkably effective against multiple manifestations of

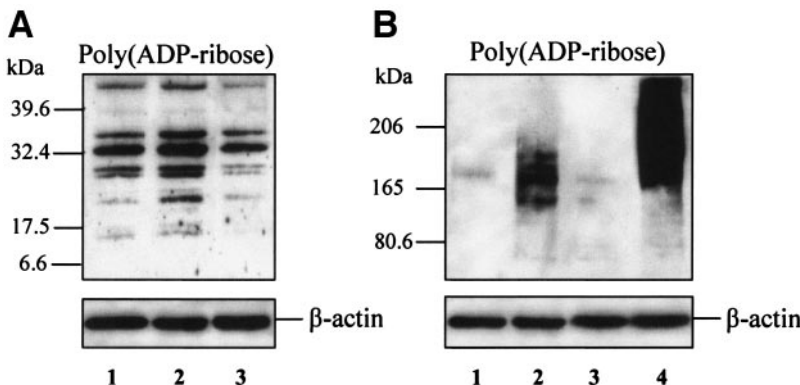


FIG. 6. A: Western blot of poly(ADP-ribosyl)ated proteins in human endothelial cells. Lane 1: cultured in normoglycemic conditions (7 mmol/l glucose); lane 2: cultured in hyperglycemic conditions (30 mmol/l glucose); lane 3: cultured in 30 mmol/l glucose plus 20 μmol/l ISO. B: Western blot of poly(ADP-ribosyl)ated proteins in HSCs. Lane 1: cultured in normoglycemic conditions (5.5 mmol/l glucose); lane 2: cultured in hyperglycemic conditions (30 mmol/l glucose); lane 3: cultured in 30 mmol/l glucose plus 20 μmol/l ISO; lane 4: cultured in 30 mmol/l glucose plus 50 μmol/l of the potent prooxidant primaquine (positive control). ISO-treated group was added to demonstrate that high glucose-induced accumulation of poly(ADP-ribosyl)ated proteins is a product of catalytic PARP-1 activity.

early PDN and may constitute a perspective approach to attain beneficial properties of PARP inhibitors and to avoid potential side effects of higher dose long-term monotherapies resulting in complete PARP inhibition.

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