Downregulation of diaphragm electron transport chain and glycolytic enzyme gene expression in sepsis

Leigh Ann Callahan and Gerald S. Supinski
Division of Pulmonary and Critical Care Medicine, Department of Medicine, Medical College of Georgia, Augusta, Georgia
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Callahan, Leigh Ann, and Gerald S. Supinski. Downregulation of diaphragm electron transport chain and glycolytic enzyme gene expression in sepsis. J Appl Physiol 99: 1120–1126, 2005; doi:10.1152/japplphysiol.01157.2004.—Cellular energy metabolism is altered in sepsis as a consequence of dysfunction of mitochondrial electron transport and glycolytic pathways. The purpose of the present study was to determine whether sepsis is associated with compensatory increases in gene expression of electron transport chain and glycolytic pathway proteins or, alternatively, whether gene expression decreases in sepsis, contributing to abnormalities in energy metabolism. Studies were performed using diaphragms from control and endotoxin-treated (8 mg·kg\(^{-1}\)·day\(^{-1}\)) rats; at 48 h after endotoxin administration, animals were killed. Microarrays and RNase protection assays were used to assess the expression of several electron transport chain components (cytochrome-c oxidase subunits Cox 5A, Cox 5B, and Cox 6A, ATP synthase, and ATP synthase subunit 5B) and of the rate-limiting enzyme for glycolysis, phosphofructokinase (PFK). Western blotting was used to assess protein levels for these electron transport chain subunits and PFK. Activity assays were used to assess electron transport chain and phosphofructokinase function. We found that sepsis evoked 1) a downregulation of genes encoding all examined electron transport chain components (e.g., cytochrome-c oxidase 5A decreased 45 ± 7%, \(P < 0.01\)) and PFK (\(P < 0.001\), 2) reductions in protein levels for these electron transport chain subunits and PFK (\(P < 0.05\) for each), and 3) decreases in mitochondrial state 3 respiration rates and phosphofructokinase enzyme activity (\(P < 0.01\) for each comparison). We speculate that these sepsis-induced reductions in the expression of genes encoding critical electron transport and glycolytic proteins contribute to the development and persistence of sepsis-induced abnormalities in cellular energy metabolism.

phosphofructokinase; mitochondria; endotoxin; muscle

THE SEPSIS SYNDROME IS COMMONLY associated with the development of severe abnormalities in tissue oxygen utilization and acid-base balance (7, 24). Specifically, peripheral tissue oxygen extraction is often reduced, with the paradoxical finding of an elevated venous oxygen level in the presence of markedly increased tissue lactate acid generation. It has been argued that these disturbances are due, at least in part, to alterations in microvascular blood flow distribution, which result in hyper-perfusion of some tissue areas and underperfusion of others (12). Recent work suggests, however, that intrinsic alterations in tissue metabolism may be the major factor limiting cellular oxygen utilization in this syndrome (2, 5, 10). These studies have suggested that alterations in metabolic processes that impact cellular ATP generation (i.e., mitochondrial dysfunction, alterations in glycolytic pathway activity) contribute to abnormalities in tissue oxygen utilization and tissue function in sepsis (1, 4, 13, 25, 28). In fact, one recent report found significant derangements in skeletal muscle mitochondrial function in many critically ill intensive care unit patients with sepsis, with poor mitochondrial function a strong predictor of multiorgan failure and death in this patient population (2).

There has been little study of the effect of sepsis on the expression of genes encoding for enzymes involved in energy metabolism. In theory, altered metabolic pathway gene expression in this syndrome could have several effects. On the one hand, expression of genes encoding proteins inhibited, damaged, or depleted in the sepsis syndrome might increase, enabling compensatory production of new enzymatic proteins involved in electron transport and glycolysis, thereby assisting in recovery from sepsis. Alternatively, expression of genes encoding proteins involved in metabolism could decrease, reducing the functional activity of key metabolic enzymes.

The purpose of the present study was to examine the effect of sepsis on expression of the gene encoding for skeletal muscle phosphofructokinase (PFK), a rate-limiting enzyme of the glycolytic pathway, and representative genes expressing electron transport chain components. As a first step, we used microarrays to compare expression of these genes in diaphragm samples taken from control animals and animals receiving endotoxin for 48 h. We next used an RNase protection assay to validate the microarray results. We then measured cellular levels of the proteins encoded by these particular genes. Finally, we assessed PFK activity and electron transport chain functional activity for diaphragm samples from control and endotoxin-treated animals and correlated alterations in mRNA levels with physiological capacity.

METHODS

Experimental protocol. Studies were performed on adult male Sprague-Dawley rats (250–350 g) housed according to the American Association for Accreditation of Laboratory Animal Care specifications and provided with food and water ad libitum. All protocols were approved by the institutional animal care and use committee at the Medical College of Georgia. Three groups of studies were performed. In the first group, we compared gene expression in diaphragms of control (\(n = 7\)) and endotoxin-treated (\(n = 7\)) animals. Because previous work by our group indicates that at least 36–48 h are required for the manifestations of endotoxin administration on diaphragm energy metabolism to become pronounced (4), diaphragm samples for RNA extraction and assessment of gene expression were obtained at 48 h after animals were injected intraperitoneally with either saline (controls, 1 ml of saline/day) or endotoxin (injected with 8 mg·kg\(^{-1}\)·day\(^{-1}\) of endotoxin, Escherichia coli lipopolysaccharide).

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Sigma Chemical, St. Louis, MO). Both groups were also given saline (60 ml/kg \( \cdot \) day \(^{-1} \)) injected subcutaneously to prevent dehydration. At the time of organ harvest, animals were deeply anesthetized with pentobarbital (60 mg/kg), the aorta was perfused with diethylpyrocarbonate-treated water (to inactivate RNAse), diaphragms were excised, and samples were stored for RNA extraction.

In a second group of studies, additional animals were killed to assess the time course of PFK gene expression after endotoxin administration by using an RNAse protection assay. For these studies, animals were killed at 6 h (\( n = 3 \)), 12 h (\( n = 3 \)), 24 h (\( n = 3 \)), 36 h (\( n = 3 \)), and 48 h (\( n = 3 \)) after initiation of endotoxin administration (at 8 mg \( \cdot \) kg \(^{-1} \cdot \) day \(^{-1} \)) intraperitoneally) and diaphragms were removed for RNA extraction. RNA from saline-treated animals (\( n = 3 \)) was also used to assess control levels of PFK expression using the RNAse protection assay.

In a third group of studies, diaphragm samples were collected from saline-injected control (\( n = 8 \)) and endotoxin-treated (\( n = 8 \)) animals; these latter muscle samples were used for determination of muscle protein levels of PFK, protein levels for five mitochondrial electron transport pathway subunits [i.e., cytochrome-c oxidase subunits 5A, 5B, and 6A (Cox 5A, Cox 5B, and Cox 6A, respectively) and ATP synthase subunits 5H and 5B (ASH and ASB, respectively)], assay of PFK enzyme activity, and measurements of diaphragm mitochondrial respiration. For this last group of experiments, all animals were killed at 48 h after initiation of endotoxin administration (8 mg \( \cdot \) kg \(^{-1} \cdot \) day \(^{-1} \)) or saline (1 ml/day intraperitoneally). At the time of death, diaphragms from these animals were flushed with ice-cold assay buffer during harvesting.

**Microarray determination of gene expression.** Microarray determinations were carried out using the Clontech Atlas microarray (Atlas Rat 1.2 Array according to the manufacturer’s specifications (Clontech, Palo Alto, CA)). In brief, total RNA was extracted from muscle samples by use of Trizol reagent (Invitrogen, Carlsbad, CA). RNA was then DNase treated and analyzed by gel electrophoresis to verify RNA purity. Radiolabeled cDNA probes were then synthesized from total RNA and hybridized to the Atlas Microarray. Atlas array membranes were then washed and analyzed by exposure to X-ray film. Completed radiographs were scanned and analyzed with Atlas software. RNA samples were assessed in pairs (one control, one endotoxin-treated sample), and expression ratios for genes of interest were calculated for each sample pair. Conventional statistical analysis of paired expression results was carried out using a paired t-test with Bonferroni’s correction for multiple comparisons. Because the present study was designed to examine only genes important in energy metabolism, the following genes were assessed: PFK, Cox 5A, Cox 5B, Cox 6A, ASH, and ASB. As a control, expression of hypoxanthine-guanine phosphoribosyl transferase (HRPT), a conventional “housekeeping” gene, was also examined.

**RNAse protection assay.** An RNAse protection assay was used to confirm the most important finding of the microarray results (i.e., a marked endotoxin-induced reduction in PFK gene expression). For this assay, a MAXscript in vitro transcription kit (Ambion, Austin, TX) was used to generate biotin-labeled RNA from template PFK DNA. A HybSpeed RPA hybridization kit (Ambion) was then used to hybridize total RNA from experimental diaphragm samples to biotin-labeled PFK RNA followed by RNAse digestion of single-stranded RNA. RNA hybrids were then isolated by gel electrophoresis and transferred to nylon membrane. A BrightStar Biotin detection kit (Ambion) was used to visualize probe-target RNA complexes; membranes were scanned and bands were analyzed by computer-assisted densitometry.

**Western blotting for determination of protein levels.** Western blotting was employed to measure diaphragm levels of PFK, Cox 5A, Cox 5B, Cox 6A, ASH, and ASB. Antibody for PFK determinations was kindly provided by Dr. Robert G. Kemp (Department of Biochemistry, Rosalind Franklin University of Medicine and Science, North Chicago, IL). Antibodies for electron transport chain subunit detection were obtained from MitoScience LLP (Eugene, OR). As a loading control, actin levels were assessed by using antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

For these determinations, muscle homogenates were diluted with an equal volume of loading buffer (126 mM Tris-HCl, 20% glycerol, 4% SDS, 1.0% 2-mercaptoethanol, 0.005% bromophenol blue, pH 6.8) and loaded onto Tris glycine polyacrylamide gels, and proteins were separated by electrophoresis (Novex Minicell II, Carlsbad, CA). Reference protein standards with known molecular weights were run in conjunction with experimental samples. Proteins were then transferred to polyvinylidene difluoride membranes. After electroblotting, they were blocked for 1.0 h at room temperature in PBS containing 5% milk and 0.05% Tween 20. After washing in PBS Tween, membranes were incubated overnight at 4°C with primary antibodies to targeted proteins. Membranes were then washed extensively and subsequently incubated with anti-mouse horseradish peroxidase-conjugated IgG 1:2,000 for 1 h at room temperature. Antibody binding to proteins was detected by enhanced chemiluminescence (Perkin Elmer Sciences, Boston, MA). Gel densitometry was performed using a Micropatch 200 (Carson, CA) and Un-Scan-It software (Silk Scientific, Orem, UT).

**PFK activity assay.** A standard enzyme-linked reaction assay (6, 14, 15, 18, 20, 21) was used to assess PFK activity for diaphragm muscle samples. In brief, fructose-6-phosphate and ATP were added to muscle homogenates, generating fructose 1,6 diphosphate. Aldolase, triosephosphate isomerase, and glycophosphate were also added, resulting in the oxidation of 2 mol of NADH for each mole of fructose diphosphate formed. NADH oxidation rates were monitored by carrying out the assay in a spectrophotometer set to 340 nm. PFK activity units were calculated by multiplying the optical density change per minute by 0.0804.

**Assessment of mitochondrial oxidative phosphorylation.** After removal from animals, tissues were rinsed in cold isolation buffer (180 mM KCl, 5 mM MOPS, 2 mM EGTA, pH 7.25 at 4°C), blotted dry, weighed, and placed in fresh buffer. After mincing with scissors, muscle pieces were homogenized (7 s) by use of a Polytron homogenizer set at half speed. The homogenate was then filtered through two layers of cheesecloth into a clean centrifuge tube and centrifuged at 600 g for 7.5 min at 4°C. The resulting supernatant was centrifuged at 5,000 g for 10 min at 4°C. The isolated mitochondrial pellet was resuspended in isolation buffer to yield a final mitochondrial protein concentration of 10–30 mg/ml (assessed with the Bio-Rad protein assay). State 3 and state 4 mitochondrial oxygen consumption were measured for mitochondrial suspensions by using a Clark-type oxygen electrode (Instech, Plymouth Meeting, PA) as previously described (4, 16).

**Statistical analysis.** Data are expressed as means ± SE. Statistical calculations were carried out using SigmaStat (SPSS, Chicago, IL). Comparison of gene expression between control and endotoxin-treated groups was done using paired t-tests with a Bonferroni’s correction for multiple comparisons (Geo Accession Number GSM32265). Examination of the time course of PFK expression using the RNAse protection assay was accomplished by ANOVA analysis; a Bonferroni’s post hoc test was used to determine significance for individual experimental groups. A \( P \) value < 0.05 was taken as indicating statistical significance. Data are reported as means ± SE.

**RESULTS**

Microarray determination of gene expression for samples form control and endotoxin-treated animals. Figure 1 displays typical Clontech microarrays generated with RNA isolated from diaphragm muscle samples from control and endotoxin-treated animals. Further analysis of array data was carried out by using AtlasImage 1.5 Software (Clontech) and was focused on genes encoding proteins involved in intermediary metabolism. This microarray includes assessment of five genes coding.
proteins that are components of the mitochondrial electron transport chain; results of the analysis of mRNA for these particular electron transport chain genes are shown in Fig. 2. Endotoxin administration induced reductions in the expression of all mitochondrial genes included in the microarray as follows: 1) 33 ± 10% reduction in Cox 5B (P < 0.05), 2) 23 ± 7% reduction in Cox 6A (P < 0.05), 3) 45 ± 7% reduction in Cox 5A (P < 0.005), 4) 56 ± 12% reduction in ASH (P < 0.01), and 5) 26 ± 8% reduction in A5B (P < 0.05).

We also assessed expression of PFK, the rate-limiting enzyme in glycolysis. Endotoxin administration resulted in large reductions in phosphofructokinase expression, as shown in Figs. 1 and 2 (expression of this gene was 77 ± 6% lower for samples from endotoxin-treated animals compared with the controls, P < 0.001).

RNAse protection assay results. To validate the findings of the microarray, we performed an RNAse protection assay examining PFK expression using this alternative methodology. As a control, 28S ribosomal RNA levels were also assessed when performing this assay. For this determination, we also examined the time course of expression of this particular gene to determine how soon after endotoxin administration expression declined. PFK expression remained relatively constant over the first 24 h but fell dramatically at the 36- and 48-h time points after endotoxin administration, as shown for representative samples in Fig. 3. Mean RNAse expression data are shown in Fig. 4; on average, PFK expression decreased by 87 ± 11%, P < 0.001 (this correlated well with the 77% decrease noted in the microarray analysis). Levels of the control, 28S ribosomal RNA, were unchanged across the lanes, indicating equal loading of all lanes.

PFK and electron transport chain subunit protein levels. Representative Western blots for determination of protein levels of PFK and five electron transport chain subunits from
control and endotoxin-treated diaphragm samples are presented in Fig. 5. Endotoxin administration was associated with an appreciable reduction in diaphragm protein levels for both PFK and all five electron transport chain protein subunits (Cox 5A, Cox 5B, Cox 6A, A5H, A5B) in these blots. Levels of a loading control protein (actin) were not different between control and endotoxin samples. Group mean data for protein determinations are displayed in Fig. 6. Mean protein levels declined significantly for both PFK and all electron transport chain components tested, falling 25–59% below levels observed for samples from control animals (P < 0.05 for comparison with baseline, control levels). 28S ribosomal levels were unchanged over time.

Functional analysis of enzyme activities. Endotoxin administration elicited significant reductions in both mitochondrial respiration rate and PFK functional activity as shown in Table 1 and Fig. 7. Mitochondrial oxidative phosphorylation, assessed by measurement of state 3 respiration, declined by 48% in response to endotoxin administration (Table 1, P < 0.01). This decline in state 3 respiration rate was not associated with an increase in state 4 or a reduction in the amount of ADP utilized per nanoatom O consumed (ADP/O ratio). We also found that PFK functional activity decreased by an average of 27% in response to endotoxin administration (Fig. 7, P < 0.008).

DISCUSSION

The sepsis syndrome is associated with the development of significant alterations in tissue metabolic function. Clinically, patients with this syndrome often demonstrate increased lactic acid generation and other evidence of tissue hypoxia despite the presence of a hyperdynamic circulation with a normal to increased level of cardiac output (7, 24). This contrasts with other clinical conditions, such as cardiogenic and hypovolemic shock, in which lactic acidosis develops as a consequence of reduced tissue perfusion secondary to reductions in cardiac output. This apparent paradox has lead to the concept that sepsis represents a state of “cytopathic hypoxia” in which the anaerobic pathways of ATP generation are recruited in tissues even though bulk delivery of blood and oxygen to these tissues remains adequate (2, 7, 8).

Several processes may contribute to the development of these metabolic derangements, including alterations in intermediary metabolism that limit ATP generation (1, 4, 12). A number of sepsis-induced alterations in cellular energy pathways have been described, with reports of sepsis-induced alterations in pyruvate dehydrogenase activity (13, 28), reduc-
tions in Krebs cycle enzyme function (27), inhibition of electron flow along the electron transport chain (4), uncoupling of oxidative phosphorylation (1), and impaired transport of ATP out of mitochondria by the sarcomeric creatine kinase shuttle (3). In keeping with this concept, one recent report found that the level of skeletal muscle mitochondrial impairment in critically ill patients with sepsis predicted survival, with higher degrees of mitochondrial dysfunction associated with a greater mortality (2). Various mechanisms have been proposed to explain these sepsis-induced alterations in energy pathway activities (4, 9, 13, 17). One process by which sepsis is thought to alter the function of proteins is via the generation of toxic species (e.g., free radicals, peroxynitrite, nitric oxide) that react with and alter the functional capacity of enzymes (4, 17). Sepsis also evokes cytokine-mediated activation of cell signaling pathways and resultant kinase-mediated phosphorylation of cellular enzyme systems (13).

All of the above mechanisms, however, act by altering the function of existing transport proteins and enzymes. Another manner in which metabolic function could be altered in sepsis is via an effect on gene transcription and translation, thereby increasing or decreasing synthesis of critical metabolic enzymes. There has been little work examining this issue in the past, however, with previous studies concentrating only on alterations in liver (25). Teleologically, one might expect sepsis to evoke a compensatory increase in genes encoding critical components of the metabolic machinery, thereby replacing proteins damaged by cellular toxins and allowing cells to adapt to the stress of sepsis by augmenting the cellular capacity to generate ATP aerobically (25). Alternatively, energy pathway gene expression in sepsis may decrease and may represent a pathological cellular response to cytokines. Such reductions in gene expression could make it difficult for cells to compensate for damage to energy pathway enzymes and, in the extreme, could directly cause or contribute to the energetic alterations seen in sepsis.

The present findings would suggest that the initial skeletal muscle response to sepsis is most consistent with the latter possibility, with our data indicating a significant sepsis-induced reduction in mRNA levels for genes encoding a number of important metabolic pathway enzymes. This was not part of a generalized downregulation of genes, as expression of a housekeeping gene (i.e., we examined HRPT in the present study) was not altered. Because our analysis was confined to measurement of mRNA levels for the genes examined, we cannot exclude the possibility that the effects of sepsis on mRNA levels of these genes was primarily the result of an effect to reduce mRNA stability rather than to reduce transcription rates for these genes. In either case, however, these sepsis-induced reductions in mRNA levels for PFK and several electron transport chain proteins would be expected, other factors being equal, to reduce translation rates for these particular proteins.

Table 1. Mitochondrial parameters

<table>
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<tr>
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<th>Control</th>
<th>Endotoxin-Induced Sepsis</th>
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<tr>
<td>State 3 respiration, natoms O/min⁻¹·mg⁻¹</td>
<td>220±3</td>
<td>115±5*</td>
</tr>
<tr>
<td>State 4 respiration, natoms O/min⁻¹·mg⁻¹</td>
<td>40±5</td>
<td>34±6</td>
</tr>
<tr>
<td>Respiratory control ratio</td>
<td>7.0±0.2</td>
<td>3.4±0.2*</td>
</tr>
<tr>
<td>ADP/O</td>
<td>3.2±0.1</td>
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Values are means ± SE. ADP/O, ratio of ADP utilized per nanoatom O consumed. *P < 0.01 compared with control.

Fig. 6. Group mean data for PFK and electron transport chain protein levels comparing samples from control (solid bars) and endotoxin-treated animals (open bars). Endotoxin induced sepsis elicited a significant reduction in protein levels for PFK and all 5 electron transport chain subunits (*P < 0.05 comparing control with septic conditions).

Fig. 7. PFK activity assessed using an enzyme-linked in vitro assay. Samples obtained from animals at 48 h after administration of endotoxin to induce sepsis had significantly reduced levels of PFK activity compared with controls (*P < 0.01).

Fig. 8. Group mean data for PFK and electron transport chain protein levels comparing samples from control (solid bars) and endotoxin-treated animals (open bars). Endotoxin induced sepsis elicited a significant reduction in protein levels for PFK and all 5 electron transport chain subunits (*P < 0.05 comparing control with septic conditions).
tional activity, paralleling the sepsis-induced reductions in PFK mRNA and protein levels. To our knowledge, the present data provide the first demonstration that PFK gene expression and functional activity can acutely undergo major changes in response to a physiological stress. Chronic reductions in PFK activity are present, however, in Tarui’s disease (22). PFK is the rate-limiting enzyme for glycolysis, and loss of activity of this enzyme reduces the capacity of cells to utilize glucose to generate pyruvate. Patients with Tarui’s disease typically suffer from impaired exercise capacity and reduced muscle function (22). The functional reduction in PFK activity that we observed in sepsis would be expected to produce effects similar to those observed in Tarui’s disease, reducing entry of glucose into metabolic pathways, ATP-generating capacity, and muscle function.

We also found reductions in state 3 respiration rates for mitochondria isolated at 48 h after the initiation of endotoxin administration. The state 3 respiration rate measures the maximal response of oxidative phosphorylation to ADP stimulation. We found that this index fell in parallel with concomitant reductions in mRNA and protein levels for several electron transport chain components. The present finding of a reduction in diaphragm skeletal muscle mitochondrial function during the development of sepsis is in keeping with several recent reports demonstrating sepsis-induced reductions in muscle mitochondrial capacity for generation of ATP (1, 4). The present report is the first, however, to show a sepsis-induced reduction in mRNA encoding skeletal muscle mitochondrial electron transport chain proteins. The fact that sepsis leads to a reduction in both the functional activity of the rate-limiting enzyme in glycolysis (PFK) and mitochondrial electron transport chain capacity indicates that sepsis is associated with a generalized reduction in the capacity of muscle to generate ATP by both aerobic and anaerobic pathways.

Potential effects of sepsis-induced metabolic alterations on diaphragm function. Numerous recent reports have indicated that significant diaphragmatic dysfunction occurs during the development of sepsis (23, 26). The magnitude of the reduction in diaphragm force-generating capacity evoked by this stress can be profound, and, in one animal study, it caused death due to respiratory failure if mechanical ventilation to support respiration was not provided (11). The mechanism by which sepsis producing alterations in the functional capacity of the diaphragm and other skeletal muscles has been the subject of much study, with a variety of subcellular sites of damage reported in animal models of sepsis. It has been suggested, moreover, that diaphragm mitochondrial dysfunction may play an important role in potentiating sepsis-induced muscle failure (1). According to this view, reductions in metabolic function may result in a shift in the equilibria between creatine phosphate, creatine, ATP, ADP, and phosphate such that higher levels of creatine, phosphate, and ADP result during periods of muscle activity. High phosphate levels have a direct and profound effect to reduce contractile protein function, shifting the force-pCa relationship of the contractile proteins (19). By this mechanism, sepsis-induced reductions in respiratory muscle high-energy phosphate compound generation could significantly reduce the functional capacity of these muscles. The present results are in keeping with this possibility, providing evidence that anaerobic as well as aerobic pathways of diaphragm energy metabolism may be compromised in sepsis and that sepsis-related alterations in gene expression may perpetuate these metabolic alterations.

ACKNOWLEDGMENTS

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GRANTS

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