Acute Regulation of Fatty Acid Oxidation and AMP-Activated Protein Kinase in Human Umbilical Vein Endothelial Cells

Zeina Dagher, Neil Ruderman, Keith Tornheim, Yasuo Ido

Abstract—It is generally accepted that endothelial cells generate most of their ATP by anaerobic glycolysis and that very little ATP is derived from the oxidation of fatty acids or glucose. Previously, we have reported that, in cultured human umbilical vein endothelial cells (HUVECs), activation of AMP-activated protein kinase (AMPK) by the cell-permeable activator 5-aminoimidazole-4-carboximide riboside (AICAR) is associated with an increase in the oxidation of \(^3\)H-palmitate. In the present study, experiments carried out with cultured HUVECs revealed the following: (1) AICAR-induced increases in palmitate oxidation during a 2-hour incubation are associated with a decrease in the concentration of malonyl coenzyme A (CoA) (an inhibitor of carnitine palmitoyl transferase 1), which temporally parallels the increase in AMPK activity and a decrease in the activity of acetyl CoA carboxylase (ACC). (2) AICAR does not stimulate either palmitate oxidation when carnitine is omitted from the medium or oxidation of the medium-chain fatty acid octanoate. (3) When intracellular lipid pools are prelabeled with \(^3\)H-palmitate, the measured rate of palmitate oxidation is 3-fold higher, and in the presence of AICAR, it accounts for nearly 40% of calculated ATP generation. (4) Incubation of HUVECs in a glucose-free medium for 2 hours causes the same changes in AMPK, ACC, malonyl CoA, and palmitate oxidation as does AICAR. (5) Under all conditions studied, the contribution of glucose oxidation to ATP production is minimal. The results indicate that the AMPK–ACC–malonyl CoA–carnitine palmitoyl transferase 1 mechanism plays a key role in the physiological regulation of fatty acid oxidation in HUVECs. They also indicate that HUVECs oxidize fatty acids from both intracellular and extracellular sources, and that when this is taken into account, fatty acids can be a major substrate for ATP generation. Finally, they suggest that AMPK is likely to be a major factor in modulating the response of the endothelium to stresses that alter its energy state. (Circ Res. 2001;88:1276-1282.)

Key Words: glucose transport ■ AICAR ■ malonyl CoA ■ acetyl CoA carboxylase ■ fuel metabolism

Increased levels of long-chain fatty acids have been implicated in causing endothelial cell dysfunction and pathology.\(^1,2\) In various vascular cells and intact blood vessels, fatty acids have been shown to affect ion transport,\(^3\) the activity of protein kinase C\(^4–6\) and other kinases,\(^7,8\) and the production of reactive oxygen species.\(^9,10\) In addition, they have been reported to decrease nitric oxide production and thus endothelium-dependent vasodilation\(^6,11,12\) and increase the expression of leukocyte adhesion molecules in the endothelium.\(^13\) Despite this, little is known about the regulation of fatty acid oxidation by the endothelium. Indeed, it is generally held that fatty acid oxidation accounts for very little of the ATP generated by these cells.\(^14–16\)

An enzyme that has been implicated in regulating fatty acid oxidation in heart,\(^17\) liver,\(^18\) and skeletal muscle during exercise\(^19–22\) is AMP-activated protein kinase (AMPK). When the energy state of these tissues is compromised, AMPK is both allosterically and covalently activated by events set in motion by an increase in the AMP:ATP ratio. Once activated, it stimulates fatty acid oxidation by phosphorylating and inhibiting acetyl coenzyme A (CoA) carboxylase (ACC)\(^20,21\) and possibly phosphorylating and activating malonyl CoA decarboxylase.\(^23\) This results in a decrease in the concentration of malonyl CoA, an inhibitor of carnitine palmitoyl transferase 1 (CPT1), the enzyme that controls the transfer of long-chain fatty acyl CoAs into the mitochondria where they are oxidized. A similar chain of events has been demonstrated in cardiac and skeletal muscle after incubation or perfusion with the cell-permeable AMPK activator 5-aminoimidazole-4-carboximide riboside (AICAR).\(^17\)

In an earlier publication, we demonstrated that AMPK is present in human umbilical vein endothelial cells (HUVECs), and that when it is activated by incubation with AICAR for 2 hours, ACC activity decreases and the oxidation of radioac-
tive palmitate added to the medium is increased.\textsuperscript{24} Even under these circumstances, however, fatty acid oxidation seemed to be a minor fuel of the endothelium, accounting for only 14\% of calculated ATP generation. In addition, the increase in fatty acid oxidation did not compensate for the decrease in glycolytically generated ATP caused by AICAR, and as a result, calculated ATP production was diminished by 33\%. Because cellular ATP levels were, if anything, increased in these cells, this raised the possibility that measurement of the oxidation of a fatty acid added to the medium-underestimated cellular fatty acid oxidation. This article extends these earlier studies. The roles of carnitine and malonyl CoA in the regulation of fatty acid oxidation in HUVECs are delineated, and the quantitative importance of fatty acid oxidation to ATP generation is reassessed, taking into account the contribution of fatty acids derived from intracellular as well as extracellular sources. In addition, the effects of glucose deprivation, which similar to AICAR increases AMPK activity, on ACC activity, malonyl CoA concentration, and fatty acid oxidation were examined. The results indicate that the AMPK–ACC–malonyl CoA–CPT1 mechanism for regulating long-chain fatty acid oxidation operates in the endothelium under physiological conditions. They also strongly suggest that fatty acids can be a major fuel of the endothelium and that, at least over brief periods of time, they can be derived predominantly from intracellular stores.

Materials and Methods

Materials
All reagents were purchased from Sigma unless otherwise noted. Radioactive chemicals were obtained from NEN Life Science Products and cells and culture materials from Clonetics.

Cell Culture and Incubation Conditions
HUVECs, passages 3 through 5, were grown in EBM\textsuperscript{2} media (Clonetics) in a 37°C, 5\% CO\textsubscript{2}/95\% air incubator and were used that, at least over brief periods of time, they can be derived predominantly from intracellular stores.

Oxidation Studies
Palmityate oxidation was measured on the basis of 3\textsubscript{H}\textsubscript{2}O production, as described by Moon and Rhead,\textsuperscript{25} in cells incubated with 2 \textmuCi/\textmuL 3\textsuperscript{H}-palmitate (2 \textmuCi/mL final concentration) and 0.05 mmol/L fatty acid–free albumin in Earle’s/HEPES solution.\textsuperscript{24} Cells preincubated 24 hours before the experiment with tracer amounts of 3\textsuperscript{H}-2-deoxy-D-glucose (5 \textmuCi/mL final concentration) and 0.01 mmol/L fatty acid-free albumin in Earle’s/HEPES solution, pH 7.4 at 37°C in the presence and absence of 2 mmol/L AICAR, as described previously.\textsuperscript{24}

Figure 1. Effects of carnitine, AICAR, and prelabeling on palmityate oxidation. Fatty acid oxidation was measured in HUVECs over 2 hours on the basis of 3\textsubscript{H}\textsubscript{2}O production from 3\textsuperscript{H}-palmitate (4.9 Ci/mmol) in both control (light bars) and AICAR-treated cells (dark bars). Incubation conditions were as follows: no carnitine, 50 \textmumol/L carnitine, and 50 \textmumol/L carnitine in cells preincubated with 2 \textmuCi 3\textsuperscript{H}-palmitate for 24 hours. Values are mean±SD, 6 to 12 replicate plates. Studies were carried out on 2 to 4 separate experimental days. *P<0.05, **P<0.01 vs control.

Materials and Methods

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Cell Culture and Incubation Conditions
HUVECs, passages 3 through 5, were grown in EBM\textsuperscript{2} media (Clonetics) in a 37°C, 5\% CO\textsubscript{2}/95\% air incubator and were used when confluent. Experiments were performed in 100-mm plates unless otherwise indicated. Before the experiments, cells were washed with warm PBS and then incubated for up to 2 hours with warm Earle’s/HEPES solution, pH 7.4 at 37°C in the presence and absence of 2 mmol/L AICAR, as described previously.\textsuperscript{24}

ACC and AMPK Assays
ACC and AMPK were assayed as previously described.\textsuperscript{24}

Oxidation Studies
Palmityate oxidation was measured on the basis of 3\textsubscript{H}\textsubscript{2}O production, as described by Moon and Rhead,\textsuperscript{25} in cells incubated with 2 \textmuCi/mL [9,10-\textsuperscript{3}H]-palmitic acid and unlabeled palmitate (0.1 mmol/L final concentration) and 0.05 mmol/L fatty acid–free albumin in Earle’s/HEPES solution.\textsuperscript{24} Cells preincubated 24 hours before the experiment with tracer amounts of 3\textsuperscript{H}-palmitate (2 \textmuCi/mL final concentration) were washed twice with 10 mL. Glucose and octanoate oxidation were determined in separate studies based on 14\textsuperscript{C} production and 14\textsuperscript{CO}\textsubscript{2} generation in flasks incubated under identical conditions but in the absence of cells.

Malonyl CoA Assay
Malonyl CoA was assayed based on the incorporation of tritium from NADPH into fatty acids in the presence of acetyl CoA and fatty acid synthase by a modification of the method described by Foerster and Lynen.\textsuperscript{26} Cells in 6-well plates were deproteinized in 0.6 mol/L perchloric acid (PCA) and neutralized with 2 mol/L KOH/2 mol/L KHCO\textsubscript{3}. The neutralized extracts were assayed for 60 minutes in a reaction mixture with the following final concentration: 200 mmol/L KPO\textsubscript{4} pH 7, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mg/mL BSA, 5 mmol/L acetyl CoA, 120 mmol/L NADPH, and 5 \textmuL/sample fatty acid synthase (purified from baker’s yeast as described by Karam and Arslanian\textsuperscript{27}).
the absence of carnitine, fatty acid oxidation was minimal and was not increased by AICAR. However, when 50 μmol/L carnitine was present in the medium, fatty acid oxidation was increased 3-fold in the AICAR-treated cells. In cells preincubated for 24 hours with 3H-palmitate, an additional 2- to 3-fold increase in fatty acid oxidation was observed in both AICAR-treated and nontreated cells (Figure 1). Prelabeling with 3H-palmitate for more than 24 hours did not increase measured fatty acid oxidation further (data not shown), suggesting that the labeled fatty acid in the cell and media were in equilibrium. In contrast to these findings, no effect of adding carnitine to the medium was observed on the oxidation of the 8-carbon fatty acid octanoate, nor was octanoate oxidation increased by AICAR (3.9 ± 0.9 versus 3.5 ± 0.7 nmol/mg protein/2 hours, respectively, in control and AICAR-treated cells in the presence of 50 μmol/L carnitine [n=6]). Overall, these data strongly suggest that (1) carnitine is required to demonstrate high rates of long-chain fatty acid oxidation in HUVECs and (2) the magnitude of fatty acid oxidation is underestimated unless carnitine is added to the medium and the cells are preincubated with radioactive fatty acids to label intracellular lipid pools.

Malonyl CoA
Malonyl CoA, the product of ACC, decreased by 70% in cells incubated with AICAR with the maximum decrease evident by 60 minutes (Figure 2A). The time course of changes in ACC and AMPK activity observed previously24 in similarly treated cells is shown in Figure 2B for comparison.

Glucose Oxidation and Glycolysis
Glucose oxidation occurred at approximately 0.1% to 0.25% of the rate of lactate release in accordance with previous reports5,16 (Figure 3). Neither parameter was affected by the addition of carnitine to the medium or by prelabeling the cells for 24 hours with 3H-palmitate. Incubation with AICAR caused a 40% decrease in lactate release and a 40% increase in glucose oxidation to CO2 in HUVECs incubated in the presence of carnitine as reported previously.24 AICAR had a similar effect when carnitine was omitted from the medium, suggesting that perhaps these changes were not secondary to its effect on fatty acid oxidation. No increase in glucose oxidation was observed on adding the pyruvate dehydrogenase (PDH) activator dichloroacetate (an inhibitor of PDH kinase) to the medium at a concentration that enhances glucose oxidation in skeletal muscle more than 2-fold (2 mmol/L) (data not shown).30 The addition of adenosine deaminase (5 U/mL) to the incubation media had no effect on fatty acid oxidation, lactate release, or ATP concentration in control or AICAR-treated cells (data not shown).

Calculated ATP Production
ATP production was calculated from the rates of glycolysis and glucose and fatty acid oxidation presented in Figures 1 and 3. As shown in Figure 4, glycolysis is the major source of ATP production in HUVECs, with its relative importance varying with the experimental conditions. Glucose oxidation was a minor contributor under all of the circumstances studied. For this reason, no effort was made to distinguish between CO2 generated by mitochondrial oxidation (ie, PDH and Krebs cycle) and the pentose shunt (see Discussion). In contrast, fatty acid oxidation to CO2 accounted for 14% of total ATP production in cells prelabeled with palmitate and for >37% when the prelabeled cells were treated with AICAR. As shown in Figure 4, when this corrected value for fatty acid oxidation was used, the calculated deficit in ATP production caused by the decrease in glycolysis in HUVECs...
treated with AICAR was much smaller, and it was not statistically significant.

Nucleotide Analyses and Glucose Uptake

The observation that AICAR inhibits glycolysis, independent of its effects on fatty acid oxidation, suggests that it might act directly by raising the concentration of one or more AICAR-derived nucleotides (Z-nucleotides) as it appears to do in the liver.31 To evaluate this possibility, the concentrations of ZTP, ZMP, and ATP were analyzed by HPLC. As shown in Figure 5, AICAR caused a 16% increase in the concentration of ATP in HUVECs (57.6 ± 1.5 in control cells versus 67.3 ± 1.2 nmol/mg protein), a modest accumulation of ZTP (13.9 ± 0.5 nmol/mg protein), and a much larger accumulation of ZMP (approximately 116 nmol/mg protein). Levels of other nucleotides such as GTP, GDP, and ADP were unchanged. Because the peak for AMP overlapped with that for ZMP, the precise effect of AICAR on its concentration could not be determined. Elevations in the concentrations of ZMP, ZTP, and ATP all could cause inhibition of glycolysis at phosphofructokinase.31 In keeping with this possibility, we found that AICAR does not inhibit glucose transport as evident from its lack of effect on the uptake of the nonmetabolized glucose analog, 3-O-methyl-D-glucose. However, it did diminish the net uptake of 2-deoxyglucose, a measure of glucose transport and phosphorylation, by 60% (Figure 6).

Incubation in Media Containing 0 and 30 mmol/L Glucose

The data suggest that AICAR activation of AMPK (by inhibiting ACC and causing a decrease in the concentration of malonyl CoA) increases fatty acid oxidation in HUVECs. To determine whether a similar set of events can occur in HUVECs when AMPK is activated by a stimulus other than AICAR, incubations were performed in media lacking glucose. AMPK activity was significantly increased in these cells (Figure 7), and the ACC activity and malonyl CoA concentration were decreased within 2 hours (Figures 8A and 8C). In addition, fatty acid oxidation (prelabeled cells) was increased 3-fold (Figure 8B). Interestingly, at the end of the 2-hour incubation, the concentration of ATP was not different from that of control cells (100 ± 7, 93 ± 5, and 91 ± 5 nmol/mg protein).

Figure 4. Calculated ATP production. Calculations are based on data from Figures 1 and 3. It was assumed that 1 mole of ATP is generated per mole of lactate produced (light gray), 38 moles of ATP per mole of glucose oxidized (dark gray), and 129 moles of ATP per mole of fatty acid oxidized (white). Values are mean ± SD. *P < 0.01.

Figure 5. Measurements of ZMP, ZTP, ATP, and other nucleotides. HPLC measurements were performed on neutralized PCA extracts of cells incubated with 0 (A) or 2 mmol/L AICAR (B) for 2 hours (n = 5).

Figure 6. Uptake of 3-O-methyl-D-glucose and 2-deoxy-D-glucose. A, 3H-3-O-methyl-D-glucose uptake. The uptake of this nonmetabolized glucose analog was linear for 4 minutes (data not shown) and was totally inhibited by cytochalasin B, a specific inhibitor of glucose transport (dark gray bars). See Materials and Methods. B, Uptake of 3H-2-deoxy-D-glucose, which provides a measure of glucose transport and phosphorylation, was linear for at least 8 minutes, and it was totally inhibited by cytochalasin B (data not shown). Values are mean ± SD. *P < 0.01 (n = 6).

Figure 7. Effects of 0, 5, and 30 mmol/L glucose on AMPK activity. AMPK was assayed in cells incubated for 2 hours with 0, 5, or 30 mmol/L glucose. Values are mean ± SD (n = 4). *P < 0.01.
protein in 0, 5, and 30 mmol/L glucose incubation, respectively, suggesting that the cells were able to maintain their energy state (see Discussion). When ATP generation was calculated as it was in Figure 4, however, cells incubated in glucose-free medium produced substantially less ATP than cells incubated with 5 mmol/L glucose (0.85 versus 3.32 \( \mu \)mol/h/mg protein, respectively) (see Discussion).

**Discussion**

The studies in this paper have examined how fatty acid oxidation can be acutely regulated in cultured HUVECs. The principal findings are as follows: (1) HUVECs possess a carnitine-dependent malonyl CoA–sensitive system for controlling fatty acid oxidation. (2) This system is regulated by AMP-activated protein kinase. (3) HUVECs oxidize fatty acids from both extracellular and intracellular sources. (4) When both sources are considered, fatty acid oxidation can account for a substantial percentage of calculated ATP production in these cells.

**Definitive Evidence of CPT1–Malonyl CoA Mechanism**

Data from this and an earlier study strongly suggest that the intracellular regulation of fatty acid oxidation in HUVECs occurs at the carnitine palmitoyl transferase step. Thus, we have previously shown that fatty acid oxidation is increased by incubation of HUVECs with AICAR, which activates AMPK leading to inhibition of ACC. The results of the present study indicate that these events are associated temporally with a decrease in the concentration of malonyl CoA, an inhibitor of CPT1 (Figure 2). They also show that AICAR increases palmitate oxidation only when carnitine is added to the incubation medium (Figure 1) and that it has no effect on the oxidation of the 8-carbon fatty acid octanoate, which is less dependent on or even independent of CPT1 for its entrance into mitochondria and oxidation in other tissues.

Finally, similar changes in AMPK and ACC activity and fatty acid oxidation were observed when HUVECs were incubated in a glucose-free medium, suggesting these events are not a unique effect of AICAR. Collectively, these findings, together with the demonstration that the hepatic isoform of CPT1 is present in HUVECs, provide strong evidence that fatty acid oxidation in these cells can be regulated by the malonyl CoA–CPT1 mechanism. These findings raise the question of why cultured HUVECs are carnitine deficient. The most likely answer is that this is related to the fact that most all culture media do not contain carnitine and as a result, carnitine, if present initially in cells, would be lost into the medium. When carnitine is added to the medium, net loss from cells is diminished or even reversed. Presumably, this problem is not faced by endothelial cells in vivo except under pathological conditions, because in intact organisms, carnitine is synthesized by the liver and plasma levels in the 50 \( \mu \)mol/L range generally maintained.

**Fatty Acids as a Major Fuel**

The results also indicate that fatty acids can be a major fuel of HUVECs. This is seemingly at odds with most previous studies in which the great majority of ATP generated by cultured endothelial cells was attributed to anaerobic glycolysis. Many of these studies compared only ATP generation by glycolysis with that from glucose oxidation, however. In addition, when fatty acid oxidation was assessed, such factors as the presence in the medium of carnitine and prelabeling of endogenous lipid pools were not considered. Evidence that fatty acid oxidation may be a significant fuel of the endothelium has been suggested by studies of isolated cerebral microvessel by Hingorani and Brecher and of cultured human umbilical artery endothelial cells by Hulsmann and Dubelaar. In the latter investigation, cells (2nd through 4th passage) grown in M199 containing 0.5 mmol/L free fatty acids and \(^3\)H-oleate, supplemented with 10% human serum and 10% heat-inactivated calf serum, were observed to have a high rate of fatty acid oxidation that was increased even further by the addition of carnitine to the medium. On the assumption that the mitochondria of these cells were tightly coupled, the authors calculated that fatty acid oxidation could account for as much as 70% of the ATP generated versus 30% from glycolysis. The cells and incubation conditions in these experiments were different from those described here, and such variables as the duration of the incubation and the free fatty acid:albumin ratio used were unstated; nevertheless, together with the present study, these
results strongly suggest that fatty acids can be a major fuel for the endothelium.

**Intracellular Pools**

Several other aspects of fuel metabolism in the endothelium deserve mention. One of these relates to the observation that, by prelabeling cells with fatty acids, we were able to obtain a much higher rate of fatty acid oxidation. The incorporation of fatty acid into intracellular triglyceride occurs at a significant rate in the endothelium, and lipid droplets in these cells have been observed morphologically. It is presumably from this triglyceride pool that the endogenous fatty acid used for oxidation is derived. Whether the importance of such intracellular pools is as great when a higher free fatty acid concentration is present in the incubation medium than that used by us (0.1 mmol/L) remains to be determined. Irrespective of this, the importance of intracellular lipids is suggested by the finding that endothelial cells deprived of glucose are able to maintain their energy state. Because lactate release from these cells is negligible (Figure 8D) and the breakdown of their glycogen stores is small, the likely endogenous fuel reservoir is intracellular triglyceride. A similar conclusion concerning intracellular triglyceride as a fuel source has been drawn by us based on earlier studies in which isolated cerebral microvessels (mainly endothelium) were unable to maintain ATP levels in a glucose-free medium when fatty acid oxidation was inhibited. It must be noted, however, that increased fatty acid oxidation does not fully account for the maintenance of ATP in HUVECs. Thus, calculated ATP production was only 1/4 as great as that of cells incubated with 5 mmol/L glucose. Whether this reflects an underestimate for fatty acid oxidation or a decrease in ATP use by these cells remains to be determined. With respect to the latter possibility, Hardie and Carling have suggested, based on studies on the liver, that AMPK maintains the energy state of a cell by both increasing ATP generation (eg, increased fat oxidation) and decreasing ATP use. The liver—ATP utilizing processes that were inhibited included the synthesis of fatty acids and cholesterol. Whether AMPK inhibits these and other ATP-requiring processes in the endothelium, and if so, what are its consequences, remain to be determined.

**Glucose Oxidation**

The finding that glucose oxidation is a minor contributor to ATP generation under all conditions studied is in agreement with the finding of many others. Furthermore, if anything, the calculated rate of ATP generation from glucose is probably an overestimate, because it has been determined that >90% of the CO2 generated from glucose in the endothelium is derived from its metabolism through the pentose phosphate shunt. The observation in the present study that incubation with dichloroacetate did not significantly increase glucose oxidation suggests that inhibition of PDH caused by its phosphorylation by PDH kinase was not responsible for the low rate of glucose oxidation in HUVECs. Unexplained is why incubation with AICAR modestly increased glucose oxidation. One possibility is that it did so by diminishing the rate of glycolysis (ie, a reversal of the Crabtree effect).

This is unlikely to be the sole mechanism, however. Thus, in studies in which HUVECs were incubated with 30 mmol/L glucose for 3 days, we have found that AICAR diminished glycolysis as it did here, but it decreased rather than increased glucose oxidation.

**Physiological Role of AMPK**

The observation that the activity of AMPK is increased in HUVECs incubated either in a glucose-free medium as well as with AICAR strongly suggests that AMPK can play a physiological role in regulating fatty acid oxidation in these cells. AMPK is present in nearly all cells, and it plays a major role in the response to such stresses as hypoxia, ischemia, hyperosmolarity, absence of glucose (in some cells), and muscle exercise. All of these stresses are thought to act by altering the AMP:ATP ratio, although the existence of an AMP-independent AMPK kinase has recently been suggested by Hardie. Finally, activation of AMPK could have significant effects in the endothelium beyond altering fuel metabolism. Thus, recent studies suggest that AMPK regulates NO synthase in both the endothelium and skeletal muscle. In addition, incubation with AICAR has been shown to inhibit the apoptosis caused by sustained hyperglycemia in cultured endothelial cells.

In summary, the results indicate that the AMPK–AC-CoA–CPTI mechanism is present in HUVECs and that it plays a key role in the regulation of fatty acid oxidation. They also indicate that the fatty acids used for oxidation may be derived from both extracellular and intracellular sources and that, when this is taken into account, fatty acid oxidation can be a major contributor to ATP generation. Finally, the data reveal that the activity of AMPK and fatty acid oxidation are increased when HUVECs are deprived of glucose and that this enables them to increase fatty acid oxidation and maintain their ATP content.

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**References**


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