Acute ethanol increases angiogenic growth factor gene expression in rat skeletal muscle

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Gavin, Timothy P., and Peter D. Wagner. Acute ethanol increases angiogenic growth factor gene expression in rat skeletal muscle. J Appl Physiol 92: 1176–1182, 2002.—Moderate ethanol consumption demonstrates a protective effect against cardiovascular disease and improves insulin sensitivity, possibly through angiogenesis. We investigated whether 1) ethanol would increase skeletal muscle growth factor gene expression and 2) the effects of ethanol on skeletal muscle growth factor gene expression were independent of exercise-induced growth factor gene expression. Female Wistar rats were used. Four groups (saline + rest; saline + exercise; 17 mmol/kg ethanol + rest; and 17 mmol/kg ethanol + exercise) were used to measure the growth factor response to acute ethanol and ethanol administration. Vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1), basic fibroblast growth factor (bFGF), Flt-1, and Flk-1 mRNA were analyzed from the left gastrocnemius by quantitative Northern blot. Ethanol increased VEGF, TGF-β1, bFGF, and Flk-1 mRNA at rest and after acute exercise. Ethanol increased resting Flk-1 mRNA. Ethanol increased bFGF mRNA independently of exercise. These findings suggest that 1) ethanol can increase skeletal muscle angiogenic growth factor gene expression and 2) the mechanisms responsible for the ethanol-induced increases in VEGF, TGFβ1, and Flk-1 mRNA appear to be different from those responsible for exercise-induced regulation. Therefore, these results provide evidence in adult rat tissue that the protective cardiovascular effects of moderate ethanol consumption may result in part through the increase of angiogenic growth factors.

Vascular endothelial growth factor; basic fibroblast growth factor; transforming growth factor-β1; Flt-1; Flk-1

EXCESSIVE ALCOHOL CONSUMPTION can produce both skeletal muscle and cardiac myopathy. A characteristic feature of chronic alcohol consumption is a wasting of skeletal muscle mass that is most apparent in fast-twitch type II muscle fibers (36). Even acute alcohol intoxication can cause reversible skeletal muscle dysfunction (16). Paradoxically, evidence from epidemiological studies has demonstrated a consistent association between alcohol intake and cardiovascular disease (CVD) (1, 9, 15). Consumption of one to two drinks per day demonstrates a protective effect against CVD, whereas the consumption of three or more drinks per day is associated with an increased risk for CVD.

Insulin resistance plays an important role in the pathogenesis of a number of other disease processes, including hypertension, dyslipidemia, and CVD. Recent evidence suggests that moderate alcohol consumption is associated with greater insulin sensitivity (7, 18). Skeletal muscle is the primary tissue that controls glucose disposal, where skeletal muscle capillarization may be the pathway for the transport of insulin across the capillary endothelium. Thus a reduction in skeletal muscle capillarization may contribute to insulin resistance.

Vascular endothelial growth factor (VEGF) is a highly conserved endothelial specific growth factor that promotes capillary growth and endothelial proliferation and migration predominantly through binding to the VEGF receptors, Flt-1 and Flk-1 (KDR in humans). In EA hy296 cells, a human umbilical vein endothelial-derived cell line, ethanol promotes the formation of tubelike structures resembling capillaries (29). Recent reports have suggested that the vascular protective effects of ethanol may result from increased expression of VEGF. Ethanol can increase VEGF expression in coronary artery vascular smooth muscle cells (CASMVC) (25). Ethanol did not have a direct effect on the proliferation of either CASVSMC or human umbilical vein endothelial cells (HUVEC), but the conditioned media from CASVSMC did increase HUVEC proliferation. In chicken embryo chorioallantoic membrane (CAM), ethanol has been reported to stimulate VEGF expression and angiogenesis (25). In the gastric mucosa, ethanol increases VEGF mRNA and protein expression, whereas neutralization with an anti-VEGF antibody significantly reduced the angiogenic response to ethanol-induced injury (28).

Ethanol can also alter the expression of other growth factors, including transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (bFGF) expression. In HepG2 cells, ethanol induces the expression of TGF-β1 (27). TGF-β1 expression is increased in macro-

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phages harvested from ethanol-treated rats. In the gastric mucosa, ethanol feeding decreased bFGF expression. It has been hypothesized that ethanol-induced inhibition of cell proliferation might result from interference with mitogenic factors such as bFGF.

It is well known that exercise reduces the risk of CVD. In skeletal muscle, endurance training results in an increase in oxidative enzymes and in the number of capillaries. Our laboratory has reported increases in oxidative enzymes and in the CVD. In skeletal muscle, endurance training increases bFGF.

It has been hypothesized that ethanol increases bFGF mRNA expression. It is known that ethanol increases bFGF mRNA expression. It is also known that ethanol increases bFGF mRNA expression after acute exercise. We demonstrate here that ethanol increases bFGF mRNA expression in response to acute exercise. Therefore, the purpose of this study was to investigate the effects of ethanol on angiogenic growth factor expression in skeletal muscle at rest and after acute exercise. We demonstrate here that 1) VEGF, TGF-β1, bFGF, and Flt-1 mRNA are increased by ethanol at rest and to a greater level after exercise; 2) resting Flk-1 mRNA is increased by ethanol; and 3) ethanol increases bFGF mRNA independently of exercise.

METHODS

This study was approved by the University of California, San Diego, Animal Subjects Committee. Female Wistar rats were used throughout the study. Mean age was 67 ± 2 (SD) days, and weight was 193 ± 11 (SD) g. All rats were first familiarized with a rodent treadmill (Omnipacer model LC-4, Omnitech, Columbus, OH) and taught to run at 20 m/min, 10° incline for 5 min, 48 h before the experimental protocol. The exercise bout consisted of 1 h of treadmill running at 20 m/min, 10° incline. At 10° inclination, the maximal treadmill running speed sustained for 2 min was determined by an incremental maximal test for rats of this age, weight, sex, and strain. The maximal aerobic speed sustained for 2 min was 40 m/min (24). Animals were housed in their cages and allowed standard rat food and water ad libitum before undertaking the study. Four treatment groups were defined with six rats in each group: 1) saline + rest, 2) saline + exercise, 3) 17 mmol/kg ethanol + rest, and 4) 17 mmol/kg ethanol + exercise. This dosage of ethanol would be expected to produce a blood alcohol level of ~100 mg/dl within 20 min after administration (36). Animals were injected intraperitoneally with either saline or ethanol 20 min before the start of rest or exercise. After completion of the exercise bout, all animals were anesthetized with 2% halothane in oxygen, and the left gastrocnemius muscles (both heads) were removed and total cellular RNA was isolated. Muscle samples were removed within 20 min after the completion of exercise. Samples were stored at −80°C until analysis.

RNA isolation and Northern analysis. The methods used for RNA isolation from rat gastrocnemius muscles and Northern blotting for VEGF, TGF-β1, bFGF, Flt-1, and Flk-1 have been described in detail previously (20). Briefly, total cellular RNA was isolated and separated by electrophoresis in a 6.6% formaldehyde-agarose gel. Fractionated RNA was transferred by Northern blot to Zeta probe membrane (Bio-Rad, Hercules, CA), cross-linked to the membrane by ultraviolet irradiation for 1 min, and stored at 4°C. The blots were then probed with oligolabeled [α-32P]deoxycytidine triphosphate DNA probes for VEGF, TGF-β1, bFGF, Flt-1, or Flk-1. Prehybridization and hybridization were performed in 50% formamide, 5× sodium chloride-sodium citrate (SSC), 10× Denhardt’s solution, 50 mM sodium phosphate, 1% SDS, and 250 μg/ml salmon sperm DNA at 42°C. Blots were washed with 2× SSC and 0.1% SDS at room temperature and 0.1× SSC and 0.1% SDS at 55°C (bFGF, TGF-β1, Flt-1, and Flk-1) or 65°C (VEGF). Blots were exposed to XAR-5 X-ray film (Eastman Kodak, New Haven, CT) by use of a Cronex Lightning Plus screen at −80°C. Autoradiographs were quantitated by densitometry within the linear range of signals and normalized to ribosomal 18S RNA levels.

Statistical treatment. A two-way analysis of variance (drug × exercise level) was used to determine differences in mRNA. After a significant F ratio, a Bonferroni post hoc analysis was used to determine significance between conditions. Significance was established at P ≤ 0.05 for all statistical sets, and data reported are means ± SE.

RESULTS

The mRNA response to ethanol and exercise are shown for VEGF (Fig. 1), TGF-β1 (Fig. 2), and bFGF (Fig. 3) as representative Northern blots (Figs. 1A, 2A, and 3A) and quantitative densitometry normalized to saline mRNA. Error bars represent SE.

Fig. 1. Representative Northern blot (A) and quantitative densitometry for the ratio of vascular endothelial growth factor (VEGF) mRNA to ribosomal 18S rRNA (B) for rats that had either rested (−) or exercised (+) for 1 h at 20 m/min, 10° incline, and had been injected with either saline (−) or 17 mmol/kg ethanol (EtOH; +). Saline + rest data were normalized to 1.0. All other data were normalized to saline + rest to allow for comparisons. EtOH and exercise increased VEGF mRNA. Error bars represent SE.
18S rRNA (Figs. 1B, 2B, and 3B), respectively. Gene expression was analyzed at rest and after a single, 1-h submaximal exercise run in the gastrocnemius muscles from animals injected with either saline or ethanol before rest or exercise. There were signiﬁcant main effects of both ethanol and exercise on VEGF (P < 0.002 and P < 0.01, respectively). There was no significant interaction between ethanol and exercise on either VEGF (P > 0.40) or TGF-β1 (P = 0.46) mRNA. However, there was a significant interaction of ethanol and exercise on bFGF mRNA (P = 0.007). Post hoc analysis revealed that exercise increased bFGF mRNA. In addition, ethanol increased bFGF mRNA independently of exercise. These results demonstrate that ethanol can increase several growth factors believed to play a role in skeletal muscle angiogenesis, similar to published reports of the effects of ethanol on VEGF in CAVSMC (25) and consistent with the suggestion that ethanol can promote cardiovascular protection. Ethanol delivery intraperitoneally at 17 mmol/kg would be expected to produce a blood alcohol content of ~100 mg/dl within 20 min after administration (37). Ardies et al. (4) have shown that acute exer-

Fig. 2. Representative Northern blot (A) and quantitative densitometry for the ratio of transforming growth factor-β1 (TGF-β1) mRNA to ribosomal 18S rRNA (B) for rats that had either rested (−) or exercised (+) for 1 h at 20 m/min, 10° incline, and had been injected with either saline (−) or 17 mmol/kg EtOH (+). Saline rest data were normalized to 1.0. All other data were normalized to saline rest to allow for comparisons. EtOH and exercise increased TGF-β1 mRNA. Error bars represent SE.

DISCUSSION

The principal findings of the present study are 1) VEGF, TGF-β1, and Flt-1 mRNA are increased by ethanol and exercise, 2) resting Flk-1 mRNA is increased by ethanol, and 3) ethanol increases bFGF mRNA independently of exercise. These results demonstrate that ethanol can increase several growth factors believed to play a role in skeletal muscle angiogenesis, similar to published reports of the effects of ethanol on VEGF in CAVSMC (25) and consistent with the suggestion that ethanol can promote cardiovascular protection. Ethanol delivery intraperitoneally at 17 mmol/kg would be expected to produce a blood alcohol content of ~100 mg/dl within 20 min after administration (37). Ardies et al. (4) have shown that acute exer-

Fig. 3. Representative Northern blot (A) and quantitative densitometry for the ratio of basic fibroblast growth factor (bFGF) mRNA to ribosomal 18S rRNA (B) for rats that had either rested (−) or exercised (+) for 1 h at 20 m/min, 10° incline, and had been injected with either saline (−) or 17 mmol/kg EtOH (+). Saline rest data were normalized to 1.0. All other data were normalized to saline rest to allow for comparisons. EtOH and exercise increased bFGF mRNA. Error bars represent SE. *Signiﬁcantly different from saline rest, P ≤ 0.05 # Signiﬁcantly different from all other conditions, P ≤ 0.05.
exercise in untrained rats only modestly increases ethanol clearance. In the United States, the legal blood alcohol concentration limit for operating a motor vehicle is usually 100 mg/dl. Thus the dosage of ethanol delivered in this study is well within the levels observed in humans after moderate alcohol consumption (32, 41). We did not observe any difference in exercise performance between the saline- and ethanol-treated groups.

Ethanol and VEGF mRNA. Recently, Gu et al. (25) demonstrated that exposure of CAVSMC to 20 mM ethanol for 18 h can increase VEGF mRNA and protein expression, whereas exposure as short as 6 h can produce significant increases in VEGF protein. Our results demonstrate that ethanol exposure in adult rat tissue can increase VEGF mRNA after exposure of only ~1.5 h. It is well known that the gastrocnemius is a mixed fiber-type muscle. In skeletal muscle, the exercise-induced increase in VEGF mRNA is localized to the subsarcolemmal region of the gastrocnemius (12). Recently, Brutsaert et al. (14) showed that, in the gastrocnemius, VEGF mRNA expression is not different between fiber types after treadmill exercise. Whether ethanol increases VEGF gene expression in nonskeletal muscle cells or alters the quantitative production of VEGF mRNA by the different muscle fiber types has not been investigated. One possible mechanism for the large increase in VEGF mRNA after ethanol and exercise may be that ethanol works through transcription pathways independent of the exercise-induced regulation of VEGF mRNA.

In CAVSMC, ethanol not only increases VEGF expression but also the expression of hypoxia inducible factor-1α (HIF-1α) mRNA (25). In exercising skeletal muscle, the results during hypoxic exercise have been equivocal. Breen et al. (12) demonstrated that VEGF mRNA expression was greater in hypoxia than normoxia. Gustafson et al. (26) reported a nonsignificant increase in VEGF mRNA during restricted blood flow exercise, whereas Richardson et al. (39) demonstrated no difference between exercise-induced VEGF mRNA in hypoxia and normoxia. In an attempt to reconcile these findings, Richardson et al. proposed that there might be some threshold intracellular myocyte PO2 below which further reductions in PO2 do not produce further increases in VEGF mRNA. Our previous results demonstrating a plateau in the VEGF mRNA response to exercise at workloads above ~50% support this contention (24) because PO2 also plateaus at ~50% of maximum during graded exercise (38). Recent work in abstract form suggests that HIF-1α protein levels are increased and translocated into the nucleus and that the DNA-binding activity increased in human skeletal muscle after exercise (2). It is not possible to distinguish whether the regulators of VEGF mRNA are
different between the ethanol-induced increase and the exercise-induced increased. However, we did not observe an interaction between the exercise and drug treatments in the present results, demonstrating an additive effect of ethanol on VEGF mRNA (Fig. 1) and suggesting that the mechanisms responsible for the ethanol-induced increase may be different from those responsible for the exercise-induced increase in VEGF mRNA. In might be suggested that ethanol may increase blood flow to skeletal muscle. However, ethanol does not increase resting skeletal muscle blood flow or steady-state exercising blood flow, although ethanol may transiently increase exercise-induced hyperemia (11).

Jones et al. (28) reported that intragastric administration of ethanol induced VEGF expression and angiogenesis in the gastric mucosa and speculated that the increase in VEGF was the result of gastric mucosal injury leading to microvascular damage and ischemia. The results of the present study, in which ethanol was administered intraperitoneally, demonstrate that ethanol can induce VEGF gene expression in tissue that is not directly exposed to ethanol during administration and thus absent of direct tissue injury, as previously demonstrated by Gu et al. (25). Jones and colleagues (29) demonstrated that both protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) were increased in the gastric mucosa after intragastric ethanol administration (29). In skeletal muscle, exercise or electrical stimulation activates the PKC (40) and MAPK (5) systems. There are no known reports demonstrating that ethanol activates either the MAPK or PKC systems in skeletal muscle.

**Ethanol, TGF-β₁, and bFGF.** Consistent with previous reports when samples were collected immediately after the completion of exercise (12, 24), we observed an increase in both TGF-β₁ and bFGF mRNA in skeletal muscle in response to exercise. Ethanol can also alter the expression of TGF-β₁ and bFGF expression (27, 33, 47). In rat skeletal muscle, ethanol increased TGF-β₁ mRNA (Fig. 2). Similar to the VEGF mRNA response in skeletal muscle, both hypoxia and exercise increase TGF-β₁ mRNA (12), with a plateau in TGF-β₁ mRNA in response to exercise intensities of 50% and greater (24). Thus the greater exercise-induced increase in TGF-β₁ mRNA with ethanol suggests that the mechanisms responsible for the ethanol-induced increase may be different from those responsible for the exercise-induced increase. Whether HIF-1 or some other regulator is responsible for the ethanol-induced increase in TGF-β₁ mRNA remains to be elucidated.

Exercise and ethanol both increased bFGF mRNA; however, there was a significant interaction between exercise and ethanol on bFGF gene expression. We observed a similar increase in bFGF mRNA after ethanol administration at rest and after exercise (Fig. 3). This suggests that the mechanisms responsible for the exercise-induced increase in bFGF mRNA may also be responsible for the ethanol-induced increase in bFGF mRNA. It has previously been demonstrated that bFGF gene expression is insensitive to hypoxia, nitric oxide (NO), or circulating angiotensin II in skeletal muscle (8, 12, 20, 21). However, resting skeletal muscle bFGF mRNA is reduced in response to prostacyclin (8). Clearly, the mechanisms responsible for the ethanol-induced increase in bFGF mRNA require further investigation.

**Ethanol and VEGF receptor mRNA.** To our knowledge, this is the first report to demonstrate that ethanol not only increases VEGF gene expression but also upregulates the expression of both VEGF receptors. Ethanol increases NO release and endothelial NO synthase expression in endothelial cells (48). NO synthase inhibition attenuates the exercise-induced increase in skeletal muscle Flt-1 mRNA (20). Thus the increase in Flt-1 with ethanol administration may result from increased NO production. This hypothesis remains to be investigated.

Ethanol also increased Flk-1 mRNA at rest. Our laboratory has previously demonstrated that Flk-1 mRNA expression is unaltered acutely after exercise (20, 23), whereas Olfert et al. (35) demonstrated that immediately after acute exercise Flk-1 mRNA can be reduced. We recently demonstrated that exercise can increase skeletal muscle Flk-1 expression between 4 and 16 h after the completion of exercise (23). The mechanisms responsible for this increase remain unknown. A reduction in circulating angiotensin II, via captopril, reduces Flk-1 mRNA independent of exercise (20). Recent work suggests that ethanol potentiates angiotensin II induced activation of MAPK (49), whereas MAPK inhibition has been shown to reduce ligand-induced expression of Flk-1 (45). Whether angiotensin II or MAPK is involved in the ethanol-induced increase in Flk-1 gene expression remains to be elucidated.

The biological activity of VEGF is produced through ligand binding to the VEGF receptors, Flt-1 and Flk-1. Homozygous mutation of either VEGF receptor gene in mice results in embryonic lethality, as a result of profound deficits in vasculogenesis. Flt-1 is crucial in the organization of the developing vasculature, whereas Flk-1 is essential for embryonic endothelial cell differentiation and vasculogenesis (19, 44). These high-affinity VEGF receptors are localized predominantly to the vascular endothelium, not only on proliferating endothelial cells but also on quiescent cells as well (17). A prerequisite for tumor angiogenesis is tumor expression of VEGF, as well as expression of Flt-1 and Flk-1 (6). Thus the coexpression of mRNA for VEGF and the VEGF receptors by ethanol is consistent with the coordinate action of VEGF.

In summary, we have demonstrated that 1) ethanol increases VEGF, TGF-β₁, bFGF, and Flt-1 mRNA at rest and during exercise; 2) ethanol increases resting Flk-1 mRNA; and 3) ethanol increases bFGF mRNA independently of exercise. These results provide evidence in adult rat tissue that the protective cardiovascular effects of moderate ethanol consumption may result in part through the increase of angiogenic growth factor expression.

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