Impact of Alcohol Consumption on the Outcome of Ischemic Stroke and Thrombolysis
Role of the Hepatic Clearance of Tissue-Type Plasminogen Activator

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Background and Purpose—Tissue-type plasminogen activator (tPA) is the only acute treatment for ischemic stroke. Unfortunately, the benefit of tPA-driven thrombolysis is not systematic, and understanding the reasons for this is mandatory. The balance between beneficial and detrimental effects of tPA might explain the limited overall efficiency of thrombolysis. Here, we investigated whether this balance could be influenced by excessive alcohol intake.

Methods—We used a murine model of thromboembolic stroke, coupled to an array of biochemical assays, near-infrared or magnetic resonance imaging scans, 2-photon microscopy, hydrodynamic transfections, and immunohistological techniques.

Results—We found that 6 weeks of alcohol consumption (10% in drinking water) worsens ischemic lesions and cancels the beneficial effects of tPA-induced thrombolysis. We accumulate in vivo and in vitro evidence showing that this aggravation is correlated with a decrease in lipoprotein receptor–related protein 1–mediated hepatic clearance of tPA in alcohol-exposed mice.

Conclusions—An efficient liver-driven clearance of tPA might influence the safety of thrombolysis after stroke. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.007143.)

Key Words: ethanol • fibrinolysis • intravital microscopy

Stroke is a major leading cause of death and disability worldwide. Although our understanding of the pathophysiology of ischemic stroke has increased, reperfusion induced by tissue-type plasminogen activator (tPA; thrombolysis) remains the only approved pharmacological acute treatment.1 Despite its overall benefit, thrombolysis is limited to a short therapeutic window (3 or 4.5 hours post onset), excluding many patients.2 Moreover, thrombolysis is associated with a threat of hemorrhage1 and possibly of neurotoxicity.1 Patients may differentially respond to tPA notably because of the pathogenesis, size, and location of the clot.4,5 This argues for the necessity of a personalized care, and underlying confounding/risk factors might be taken into consideration in such a scenario.

Alcohol consumption as a risk factor for stroke has been described to follow a J-shaped curve, where moderate drinkers (<15 g/d) have the lowest risk, abstainers and heavy drinkers (>60 g/d) the highest.7 However, only few studies have evaluated the consequences of alcohol consumption on the evolution of ischemic lesions once stroke occurs. Clinical trials have shown an elevated stroke-related mortality in ex-drinkers8,9 and a higher baseline stroke severity in patients with ischemic stroke after excessive chronic alcohol consumption.10 In experimental studies, it has been described that chronic alcohol exposure increases excitotoxic/ischemic damage.11–14 Surprisingly, to our knowledge, apart from 1 study dealing with intra-arterial thrombolysis,15,16 no studies have to date examined the consequences of previous alcohol consumption on the benefits of intravenous thrombolysis after ischemic stroke. Thus, the aims of our study were (1) to evaluate, in a model of stroke, the influence of alcoholic history on the benefit/risk ratio of thrombolysis and (2) to determine the mechanisms sustaining this potential influence.

We show that alcohol consumption (6 weeks, 10% in drinking water) prevents the beneficial effect of tPA-induced thrombolysis. This could result from the following noxious cascade of events in alcohol-exposed mice: (1) a reduced hepatic clearance of tPA injected intravenously for thrombolysis; (2) an increase in the bioavailability of circulating tPA; (3) a subsequent increase of its passage into the brain parenchyma, a
compartment where tPA is known to exacerbate brain damages. This alteration of tPA clearance after alcohol consumption is correlated with a selective, tissue-specific reduction of the levels of hepatic low-density lipoprotein receptor–related protein 1 (LRP-1, receptor implicated in the clearance of tPA[17,18]). Thus, we uncover here a critical role of the liver–brain axis in the beneficial effects of thrombolysis after stroke.

Methods
The complete details of methods used are described as online-only Data Supplement.

Ethanol Administration
Mice were housed with food and water (control group) or a 10% (v/v) alcohol solution (alcohol group) ad libitum access for 6 weeks. The average daily liquid intake was similar between both groups. Table I in the online-only Data Supplement shows body, brain, and liver net and relative weights of control and alcohol-exposed mice. We found no differences between control- and alcohol-exposed mice in any of these parameters.

Thromboembolic Focal Cerebral Ischemia and tPA-Induced Thrombolysis
We used the in situ thromboembolic stroke model consisting in injecting thrombin into the middle cerebral artery and followed by thrombolysis by tPA, as performed in the clinical setting. Brain lesion volumes were measured by magnetic resonance imaging analyses and regular thionine stainings.

Results
Alcohol Consumption Impairs the Beneficial Effect of tPA-Induced Thrombolysis After Stroke
We first investigated the impact of 6 weeks of alcohol consumption on postischemic outcome. We have formerly developed a model of focal thromboembolic stroke, which mimics several aspects of the clinical management of patients with stroke. In this model, the coagulation cascade is activated by the administration of thrombin, which triggers a fibrin-rich thrombus in the middle cerebral artery, which is spontaneously dissolved in the following hours by the endogenous fibrinolytic system,19 mimicking the human pathology.20 In addition, thrombolysis performed as for patients with stroke (10% bolus and 90% infusion >1 hour) also has a therapeutic window, with early recanalization being beneficial but not if performed too late.21,22 Whether thrombin exerts specific effects on the neurovascular unit23,24 beside clot formation in this model remains to be established.

This model was thus induced in control- or alcohol-exposed mice (ad libitum access to 10% alcohol solution for 6 weeks). Twenty minutes after stroke onset, tPA or saline were intravenously injected. Four groups were thus considered: control-saline (nonthrombolysed), control-tPA (thrombolysed), alcohol-saline (nonthrombolysed), and alcohol-tPA (thrombolysed). We performed a longitudinal study to measure cerebral lesions at both early (2 hours and 30 minutes; Figure 1A and 1B) and late (24 hours, Figure 1C and 1D) time points after ischemic stroke onset in control (no alcohol) and alcohol-exposed mice, treated or not by tPA. At 2 hours and 30 minutes post ischemic onset, MR diffusion-weighted imaging lesion volumes were already significantly bigger in nonthrombolysed mice exposed to alcohol (+115%) versus nonthrombolysed control mice (P<0.05; Figure 1A and 1B). At this early time, thrombolysed mice displayed smaller infarcts than nonthrombolysed mice, although the difference did not reach statistical significance for control mice (Figure 1A and 1B).

About lesion volumes measured 24 hours post stroke onset (Figure 1C and 1D), brain damages were still significantly higher in nonthrombolysed alcohol-exposed mice than in nonthrombolysed control mice (+78%; P<0.05).

However, although tPA-induced thrombolysis significantly reduced ischemic lesion volumes in control mice (−61.3% versus nonthrombolysed control mice; P<0.05), we observed that the initial beneficial effect of tPA-induced thrombolysis disappeared in mice exposed to alcohol. Noteworthy, the lesion volume because of progression between 2 hours and 30 minutes and 24 hours (ie, the difference between the lesion volume at 24 hours and 2 hours and 30 minutes) is 7.6-fold higher in alcohol-exposed mice receiving tPA than in control mice receiving tPA (15.36 versus 2.02 mm³, respectively).

We studied brain perfusion after stroke onset and did not observe any modification after alcohol consumption (Figure I in the online-only Data Supplement), showing no evidence of microvascular injuries which could aggravate ischemic lesion in this model, in accordance with previous works.25

Besides, none of the animals, whatever the treatment, displayed intracerebral hemorrhage or significant edema (measured as the volume of the ipsilateral over the contralateral hemispheres on thionin-stained sections). The lack of significant bleeding transformations is consistent with preclinical and clinical data, reporting a low percentage of hemorrhages in patients with stroke treated in the therapeutic window of tPA and suggesting that if tPA is administered soon enough after stroke, it does not increase the risk of bleeding.26–28 Moreover, we studied the effect of alcohol on the blood–brain barrier (BBB) and we observed that alcohol did not alter significantly the extent of BBB leakage (Figure II in the online-only Data Supplement).

Our results show that tPA-induced thrombolysis loses its beneficial effect after alcohol consumption in mice.

Our Model of Alcohol Consumption Does Not Modify Clotting Time, tPA Endogenous Levels and Activity, or the Fibrinolytic Efficiency of Exogenous tPA
We wanted to determine whether the loss of efficacy of brain protection in case of thrombolysis following stroke after alcohol consumption could be because of differences in the fibrinolytic efficiency of exogenous tPA (Figure III in the online-only Data Supplement). Doppler flowmetry, magnetic resonance imaging angiographic scores, in vitro clot lysis assays, enzyme-linked immunosorbent assay, and amidolytic activity assays converge to show that the loss of protection of thrombolysis in alcohol-exposed mice is not because of alterations of neither exogenous nor endogenous fibrinolytic activities.

Selective Reduction of Hepatic LRP-1 Levels After Alcohol Consumption
We evaluated liver damages potentially provoked by alcohol exposure in mice (Table I in the online-only Data Supplement).
There was no modification in liver weight (Table I in the online-only Data Supplement) and no change in the activated partial thromboplastin time, factor VII, antithrombin, or hematocrit between control and alcohol-exposed mice (Table I in the online-only Data Supplement). By contrast, we found a decrease in prothrombin time (n=6 mice per group; \( P < 0.001 \)), factor V (n=6 mice per group; \( P < 0.05 \)), and platelet number (n=6 mice per group; \( P < 0.001 \)); and an increase in factor VIII levels (n=6 mice per group; \( P < 0.01 \)) in alcohol-exposed mice compared with control mice (Table I in the online-only Data Supplement). These changes suggest a hepatic damage induced by alcohol exposure. We also performed additional experiments to measure plasminogen activator inhibitor type 1 (PAI-1) levels by enzyme-linked immunosorbent assay and quantitative polymerase chain reaction (Figure IV in the online-only Data Supplement). We observed that total plasmatic and hepatic mRNA PAI-1 levels are significantly reduced after alcohol consumption (n=4; \( P < 0.05 \)) or protein levels (n=4, Figure 2C; n=3, Figure 2D) in the brain cortex.

Delayed Hepatic tPA Clearance After Alcohol Consumption

We investigated whether the lack of efficacy of tPA treatment in alcohol-exposed mice was correlated with a modification of its bioavailability. Alexa555-labeled tPA (tPA555) was injected intravenously in control and alcohol-exposed mice, and blood samples were harvested at different times post injection (n=3 mice per group; Figure 3A) and subjected to SDS-PAGE to quantify fluorescent tPA (Figure 3A). To avoid the saturation of tPA555 fluorescent signal, we injected a low dose of

Figure 1. Alcohol consumption worsens ischemic stroke lesions and prevents the benefit of tissue-type plasminogen activator (tPA)-induced thrombolysis. A, Representative magnetic resonance imaging (MRI) acquired 2 hours and 30 minutes (2h30) after stroke onset. B, Quantification of lesion volumes 2h30 after stroke onset (n=7–8 mice per group; *\( P < 0.05 \); **\( P < 0.01 \)). At 2h30 post ischemic onset, lesion volumes were already significantly higher in non-thrombolysed mice exposed to alcohol for 6 weeks. C, Representative histological analyses taken at 24 hours post ischemic onset. D, Quantification of lesion volumes 24 hours after stroke onset (n=7–8 mice per group; *\( P < 0.05 \)). Final lesion volumes were significantly higher in alcohol-exposed mice. tPA-induced thrombolysis reduced ischemic lesion volumes in control mice, whereas it lost its beneficial effect in mice exposed to alcohol.
tPA555 (0.5 mg/kg). Although in control mice, the level of tPA555 peaked at 5 minutes post injection and came back to the baseline after 15 minutes, in alcohol-exposed mice, the levels of tPA remained elevated until 15 minutes post injection, and then returned to the baseline after 30 minutes (Figure 3A; *P <0.05). During the hour after tPA intravenous injection, the quantity of plasmatic tPA (total area under the curve) in alcohol-exposed mice was almost twice bigger than in control mice (Figure 3A; *P <0.05). In another series of experiments, we tested whether the tPA injected under the same conditions of thrombolysis (10 mg/kg, IV) was enzymatically active or not (n=4 mice per group; Figure 3B). Thirty-five minutes after the injection, we observed that the amidolytic activity of tPA in alcohol-exposed mice was significantly higher than in control mice (23% increase; *P <0.05; Figure 3B). Altogether, these results demonstrate that tPA not only remains longer in the bloodstream after a 6-week alcohol exposure, but that this tPA is still enzymatically active.

Because tPA is mainly cleared from the blood by the liver,17 we performed a series of experiments to study real-time tPA555 hepatic clearance using in vivo 2-photon microscopy (n=3 mice per group; Figure 3C). To stain blood vessels, we injected fluorescein isothiocyanate–dextran (green fluorescence; top panels), which is not recaptured by the liver between 0 and 60 minutes (Figure 3C, top panels). After a first acquisition (before injection panels), tPA 555 (red fluorescence) was intravenously injected and images of the liver were captured at different time points. In control mice, 5 minutes after injection, tPA555 has already started to enter into the liver parenchyma, and after 10 minutes, tPA 555 was almost exclusively present in the parenchyma (Figure 3C; tPA555 and merged panels). By contrast, in alcohol-exposed mice, tPA555 was still present in the blood vessels 10 minutes after its injection, and its passage into the liver parenchyma was notably decreased at every time point (Figure 3C; tPA555 and merged panels). In the second set of experiments, we performed immunohistological analyses in saline-perfused livers of control- and alcohol-exposed mice 60 minutes after tPA555 injection. We detected a significant decrease in tPA555 levels in the liver of alcohol-exposed mice (n=3 mice per group;
Figure 3. Delayed clearance of tissue-type plasminogen activator (tPA) after alcohol exposure. Electrophoresis gels showing Alexa555-labeled tPA (tPA555) plasmatic levels 15 minutes after tPA555 intravenous injection (0.5 mg/kg) and quantification of electrophoresis gels (A). Fifteen minutes after tPA555 injection, alcohol-exposed mice showed significantly higher blood tPA levels than control mice (n=3 samples per group; *P<0.05). Area under the curve measuring total tPA555 in the bloodstream of control and alcohol-exposed mice during 60 minutes after tPA555 intravenous injection (A; n=3 per group). Amidolytic activity of tPA injected at the same dose as in thrombolysis (10 mg/kg, IV), which is significantly increased after alcohol exposure (B; n=4 samples per group; *P<0.05). The clearance of tPA by the liver is dramatically reduced after alcohol consumption (C). Two-photon microscopy showing real-time hepatic clearance of tPA555 in control and alcohol-exposed mice. Blood vessels are visualized in green by fluorescein isothiocyanate (FITC)-dextran, before tPA555 injection. In control mice, after its intravenous injection (t=0), tPA555 rapidly begins to enter into the liver parenchyma (t=+5 minutes; tPA555 and merged photomicrograph), and its passage into the liver parenchyma is practically achieved at t=+10 minutes (merged photomicrograph). However, after alcohol consumption, the passage of tPA555 into the liver parenchyma is delayed and inefficient: tPA555 is still present in the blood vessels 10 minutes after its injection, and its passage into the liver parenchyma is notably decreased at all the time points t=+20 minutes, t=+30 minutes, t=+60 minutes (tPA555 and merged panels; n=3 mice per group). Immunohistological representative images of saline-perfused mice showing significantly less Alexa555-labeled tPA in the liver parenchyma of mice exposed to alcohol than in the liver of control mice (D) and quantification. Data are expressed as mean±SEM, and normalized versus control group (n=3 samples per group; *P<0.05). DAPI indicates 4',6-diamidino-2-phenylindole.

This decrease in hepatic tPA555 fluorescence in alcohol-exposed mice at 60 minutes matches with the decrease observed by in vivo 2-photon microscopy at the same time point in alcohol-exposed mice (Figure 3D; t=+60-minute panels). All these results clearly indicate an inefficient clearance of tPA by the liver of alcohol-exposed mice.
Reduced Hepatic Clearance of tPA After Alcohol Consumption Leads to an Increase in Blood-Derived tPA in the Brain Parenchyma

Because vascular tPA can cross the intact BBB and reach the brain parenchyma,1 we performed ex vivo near infrared imaging in the brain and the liver after intravenous injection of Alexa680-labeled tPA (tPA680) or albumin (albumin680) as a control (Figure 4), in control and alcohol-exposed mice. In control mice, tPA680 was detected almost exclusively in the liver, with only a weak amount of tPA680 in the brain tissue (n=5; Figure 4A–4D). Strikingly, tPA680 levels were dramatically lower in the liver of alcohol-exposed mice and were dramatically increased in the corresponding brain parenchyma (n=5; Figure 4A and 4B; 6-fold increase versus control mice; $P<0.001$). Using albumin680 as a control (same molecular weight than tPA), we did not found any difference between alcohol-exposed and control mice (Figure 4C and 4D). These results show that a 6-week alcohol exposure reduces the hepatic clearance of tPA, leading to an increased passage of tPA into the brain parenchyma.

Prolonged Presence of tPA in the Bloodstream Favours Ischemic Damages

Control mice were in vivo transiently transfected with a plasmid containing the tPA sequence (tPA-pLive) to experimentally increase circulating tPA levels, thus mimicking the impact of thrombolysis after a 6-week alcohol exposure. Three days after transfections, basal levels of active tPA in the liver and plasma were higher in tPA-pLive transfected mice than in control mice (transfected with an empty-pLive plasmid; Figure 5A). At that time, experimentally induced ischemic lesions were bigger in tPA-pLive transfected mice than in empty-pLive transfected mice (1.7-fold increase, $P<0.001$; n=7 mice per group; Figure 5B).

In an additional series of experiments, we injected increasing doses of tPA to mimic the plasmatic increase in tPA found after 6 weeks of alcohol exposure. Whereas the standard thrombolytic dose of tPA in mice (10 mg/kg) significantly reduced infarct volume, tPA at 20 mg/kg was not beneficial and did not reduce brain lesions ($P<0.05$; n=8–10 mice per group; Figure 5C).

Altogether, our data show that (1) thrombolysis is not beneficial after a 6-week alcohol exposure; (2) plasmatic tPA levels remain elevated longer after alcohol exposure because of a selective impairment of the efficacy of tPA clearance by hepatic LRP-1; (3) high levels of circulating tPA worsen ischemic brain damages. The increase in the bioavailability of tPA could increase its ability to cross the BBB; to reach the brain parenchyma, and consequently to unmask the deleterious effect of tPA in the brain parenchyma (Figure 6). We thus propose that an efficient liver–brain axis warranties the safety of thrombolysis after stroke.

Discussion

Here, we analyzed the consequences of a 6-week alcohol exposure on the risk/benefit ratio of tPA-induced thrombolysis during ischemic stroke in mice. To our knowledge, this is the first demonstration of a loss of benefit of tPA-mediated thrombolysis after alcohol consumption. Overall, our data suggest that this noxious effect of alcohol consumption results from the following mechanistic cascade: (1) alcohol consumption downregulates the expression of LRP-1 selectively in the liver; (2) this is correlated with an inefficient clearance of tPA by the liver; (3) eventually, the bioavailability of tPA is increased during thrombolysis, which facilitates its passage into the brain parenchyma, where it is known to promote BBB leakage and neurotoxicity. To our knowledge, this is the first report showing the influence of the liver capacity to control tPA bioavailability on the beneficial effects of thrombolysis.

As reported in rats subjected to transient focal ischemia in rats,13 we found that 24 hours post stroke onset, mice exposed...
to alcohol showed bigger lesions than control mice. In addition, our longitudinal study shows that alcohol consumption speeds up the evolution of ischemic lesions, since 2 hours and 30 minutes after stroke onset, mice exposed to alcohol already showed higher lesion volumes than control mice. Interestingly, a recent clinical study has shown that chronic ethanol consumption is associated with higher baseline stroke severity in patients with ischemic stroke. Strikingly, our results show that although tPA-driven thrombolysis is highly efficient in protecting the brain in control mice (60% protection), it loses its

**Figure 5.** Experimental increase in plasmatic tissue-type plasminogen activator (tPA) levels provokes higher ischemic lesions. **A,** Three days after hydrodynamic transfections, active tPA was present in the plasma of tPA-pLive transfected mice, whereas no tPA was found in the plasma of empty-pLive transfected mice. **B,** Representative histological analyses acquired at 24 hours post ischemic onset in control mice (drinking water) transfected with empty-pLive and tPA-pLive and quantification of lesion volumes 24 hours after stroke onset. Ischemic lesions were significantly higher in tPA-pLive transfected mice than in empty-pLive transfected mice (n=7 mice per group; ***P<0.001). **C,** Experimental design to mimic the increase of plasmatic tPA found in the alcohol-exposed mice by the injection of tPA 20 mg/kg; magnetic resonance imaging (MRI) representative images 24 hours after stroke onset; quantification of infarct volume. The additional infusion of tPA to control mice results in the loss of the beneficial effect of tPA at the standard thrombolytic dose used in mice (n=8–10 mice per group; *P<0.05).

**Figure 6.** Proposed mechanism of alcohol-induced loss of beneficial effect of tissue-type plasminogen activator (tPA)-mediated thrombolysis. **A,** In case of ischemic stroke, administration of tPA (ie, thrombolysis) (1) permits the lysis of the clot on the blood vessel and thus the repuffusion of the ischemic brain territories (2). When liver performance is unaltered (A), after its intravenous injection, tPA is rapidly recaptured by the liver (3) and its action is finished. **B,** However, in case of liver dysfunction (eg, after alcohol consumption for 6 weeks), we propose that there is an important decrease in liver lipoprotein receptor-related protein 1 (LRP-1) receptors (4) and thus tPA clearance drops off (5). tPA might then remain longer in the bloodstream (6), tPA passage into the brain parenchyma could be facilitated (6), and because of its neurotoxic effects on the brain parenchyma (7), tPA loses its beneficial effect.
beneficial effect after alcohol consumption, despite effective recanalization. Moreover, the lesion volume because of progression between 2 hours and 30 minutes and 24 hours was 7.6-fold higher in alcohol-exposed mice receiving tPA than in control mice receiving tPA, suggesting a delayed deleterious effect of tPA in alcohol-exposed mice.

We show that the loss of benefit of thrombolysis after stroke in alcohol-exposed mice is not because of a decrease in the fibrinolytic capacity of tPA, since restoration of blood flow in the ischemic hemispheres was similar in both thrombolysed groups (as shown by Doppler flowmetry and angiographic score). Importantly, our study shows that the defective brain protection is related to an impaired liver function. Among the changes observed, our model of alcohol consumption modified hemostatic parameters (decreases in prothrombin time and factor V levels, increase in factor VIII levels, but no changes in liver weight). Platelet number was also decreased, which is in accordance with the reports showing that alcohol-related liver disease commonly courses with thrombocytopenia because of a direct effect of ethanol on platelet formation and lifespan.30 Despite these altered hemostatic parameters and platelet number, we observed neither differences in the clotting time nor spontaneous hemorrhagic transformations after tPA administration between control and alcohol-exposed mice. Overall, this indicates that our protocol does not provoke strong liver dysfunction because it does not trigger hemostasis impairment, which is known to occur in patients with severe liver disorders,31 but rather provokes more subtle changes in liver function. This is in agreement with a recent comparison of different models of alcohol administration in animals32 and opens interesting clinical issues, since our findings may in fact be translated to a significant portion of patients with stroke, as they relate to moderate drinkers, not heavy alcoholics.

Alcohol-induced alterations of other organs could also contribute to the fate of brain cells after stroke.33 We have not address this in detail but in the study by Cook et al14, using a similar regimen of alcohol exposure (ad libitum access in the drinking water) but for a much longer period and with a 2-fold higher dose than ours, it was found that mice had reduced heart weight, mild steatosis, altered gut flora, increased serum levels of peptidoglycan, whereas many other parameters/ organs like alanine aminotransferase activity, pancreas, corticosterone or peripheral blood parameters were not altered.

Besides, we observed that total plasmatic PAI-1 levels are significantly reduced after alcohol consumption. This reduction in the plasmatic concentration of total PAI-1 is, at least partially, explained by a reduced expression of PAI-1 in the liver after alcohol exposure.35,36 Under normal conditions, tPA is efficiently cleared from the circulation by the liver and has a short half-life in the bloodstream (4–5 minutes).37 This issue could raise the possibility that lower plasmatic levels of PAI-1 in alcohol-treated mice may participate in the clearance of tPA. However, this seems unlikely in our model because, although different in control and alcohol-treated mice, plasmatic levels of PAI-1 remain low (=0.2 nmol/L) in both cases when compared with the plasmatic concentration of tPA reached during thrombolysis (=300 nmol/L). Therefore, it is expected that tPA after intravenous injection mainly circulates not complexed to PAI-1 in both control and alcohol-treated mice. Thus, knowing that tPA clearance normally relies on LRP-1,38 we also studied its expression levels in the liver, which interestingly, were dramatically decreased in alcohol-exposed mice. In parallel to reduced levels of hepatic LRP-1, we observed an increased bioavailability of exogenous, active tPA, with an impairment of tPA clearance by the liver: (1) exogenous tPA55 remained longer in the blood of alcohol-exposed mice than in control mice and (2) in vivo 2-photon liver microscopy showed an inefficient passage of exogenous tPA55 into the liver parenchyma after alcohol consumption. Our data add to previous observations in severe hepatic diseases (fibrosis or cirrhosis39), showing that even subtle changes in hepatic function increase tPA plasmatic levels because of reduced clearance.

We hypothesized that this delayed tPA clearance by the liver after alcohol consumption could result in a deleterious increase of tPA passage into the brain parenchyma. In favor of this hypothesis, near-infrared fluorescence imaging showed that control mice show most of the fluorescence in the liver (and not in the brain), whereas alcohol-exposed mice showed most of the fluorescence in the brain (and not in the liver). Moreover, hydrodynamic transfections used to increase tPA expression in the plasma of control mice (not exposed to alcohol) led to higher ischemic lesions in tPA-pLive transfected mice than in empty-pLive transfected mice.

Another nonexclusive mechanism that could explain the loss of beneficial effect of tPA after alcohol consumption could result from the established hyperexcitability of the brain induced by alcohol consumption/withdrawal.11 This latter is notably because of an increased expression and activity of N-methyl-D-aspartate receptors.

A limit to our findings is the lack of clinical observations supporting our hypotheses. Clinical evidence of the occurrence of tPA effects shown in animal models is challenging, but it is worth mentioning that after ischemic stroke, exogenous tPA was independently associated with seizure occurrence (a mechanism that involves tPA39) and a worse outcome at 3 months in this seizure subgroup of patients.40 In addition, brain imaging studies evidenced that exogenous tPA indeed promotes BBB leakage in patients with stroke.41

About the impact of alcohol consumption, Takada et al actually reported that alcohol habit is associated with a good outcome after local intra-arterial thrombolysis. However, the term alcohol habit was not defined, and it is important to point out that the authors used urokinase,15 another plasminogen activator which is known to be non-neurotoxic.42 In addition, to our knowledge, no clinical data have directly demonstrated an impaired clearance of tPA in humans with liver dysfunction. However, tPA levels have been shown to be increased in the plasma of patients with liver disease and 2 hypotheses have been formulated: either an increased release of tPA from endothelial cells or a reduced hepatic clearance of tPA.43,44

Conclusions

Our study shows that a 6-week alcohol exposure aggravates stroke lesions and that thrombolysis loses its beneficial effect after alcohol consumption. We propose that this loss of efficacy depends on the brain–liver connection in terms of tPA.
clearance and bioavailability. After alcohol consumption, the liver suffers a series of damages, including an impaired tPA clearance, which will profoundly affect stroke outcome, especially if the patient is thrombolysed. Increased circulating tPA levels (in both time and quantity) aggravate damages, possibly because of tPA’s neurotoxic effects in the brain parenchyma. Altogether, our original data reveal the importance of the liver–brain axis in the chances of success of tPA-induced thrombolysis during stroke. Challenges like alcohol consumption can induce subtle liver dysfunction, may profoundly impact the outcome of thrombolysed-stroke patients. In our opinion, attention should be paid to the history of each stroke patient, in terms of liver capacity of tPA clearance.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL
Impact of alcohol consumption on the outcome of ischemic stroke and thrombolysis: role of the hepatic clearance of tPA
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Supplemental Methods

Supplemental table I: Physiological and haemostatic parameters in control and alcohol-exposed mice.

Supplemental figure I: Effect of alcohol on cerebral perfusion 4h and 24h after stroke onset.

Supplemental figure II. Effect of alcohol consumption on BBB integrity following stroke, measured by Evans blue extravasation.

Supplemental figure III. No changes in fibrinolytic efficiency of exogenous tPA, endogenous tPA levels or activity.

Supplemental figure IV: Effect of 6 weeks of alcohol exposure on PAI-1 expression

Supplemental figure V: Effect of 6 weeks of alcohol consumption on mannose receptor expression.

Figure legends

Supplemental references
Supplemental methods

Ethical statement
Experiments were performed in accordance with French ethical laws (act no. 87–848; Ministère de l’Agriculture et de la Forêt) and European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals, and have been approved by the local ethical committee (authorisation code CENOMEXA 0113-03). All efforts were made to limit animal suffering. None of the experimental procedures induced animal mortality.

All experiments were performed following the ARRIVE guidelines (www.nc3rs.org.uk), including randomization of treatment as well as analysis blind to the treatment.

Mice and ethanol administration protocol
Naïve, healthy 3 months old male Swiss mice (35-40 g; Janvier, France) were used for all experiments, excepting hydrodynamic transfection experiments, for which naïve, healthy 3 months old C57BL6 male mice (20-25g; Janvier, France) were used. Mice were housed in a temperature-controlled room on a 12-hour light/12-hour dark cycle with food and water (“control” group) or a 10% (v/v) alcohol solution (“alcohol” group) ad libitum access for 6 weeks. Every two days, body weights and fluid intake were measured, and bottles were refilled with water or fresh alcohol solution to avoid potential evaporation effects. The average daily liquid intake was similar between both groups [2.4±0.1 ml/day/mouse for control group (drinking water) and 2.1±0.2 ml/day/mouse for alcohol group (drinking 10% alcohol solution)]. Blood alcohol levels (BAL) were measured the day of stroke induction. We did not find any significant correlation between BAL and brain lesion size in alcohol-exposed mice receiving tPA or saline (data not shown). Table I shows body, brain and liver net and relative weights of control and alcohol-exposed mice. We found no differences between control and alcohol-exposed mice in any of these parameters.

Thromboembolic focal cerebral ischemia and tPA-induced thrombolysis
Animals were deeply anesthetized with isoflurane 5% and, thereafter, maintained with 2.5% isoflurane in a 70%/30% mixture of NO2/O2. A catheter was inserted into the tail vein to allow intravenous administrations. Rectal temperature was maintained at 37±0.5°C throughout the surgical procedure using a feedback-regulated heating system. Randomisation was previously prepared by another member of the laboratory (independent from the experiments) who brought the mice to the surgeon and gave him the tubes with the treatments. Surgeries were performed by an experimenter blind-to animal group (control or alcohol-exposed mice) and blind-to treatment (saline or tPA).

We used the in situ thromboembolic stroke model, previously described (supplemental reference 1). Briefly, mice were placed in a stereotaxic device, a small craniotomy was performed, the dura was excised, and the middle cerebral artery (MCA) was exposed. A pipette was introduced into the lumen of the MCA and 1 µL of purified murine alpha-thrombin (0.75 UI; Enzyme Research Labs) was pneumatically injected to induce the in situ formation of a clot. The pipette was removed 10 minutes after, when the clot had stabilized. To induce thrombolysis, tPA [10 mg/kg and 20 mg/kg in the dose-response study; Actilyse (Boehringer Ingelheim)] was intravenously injected (tail vein, 10% bolus, 90% perfusion during 40 minutes) 20 minutes after the injection of alpha-thrombin. The control group received the same volume of saline (200 µL) under identical conditions.

After surgery, mice were returned into cages with half-fresh bedding material and any sign of pain or distress (like vocalizations, absence of grooming, prostration, locomotion, interaction
with conspecifics) was checked regularly. No animal had to be euthanized because of suffering.

**Magnetic Resonance Imaging (MRI) analyses**

MRI analyses were performed on ischemic mice 2h30 after stroke onset. Experiments were carried out on a Pharmscan 7T (Bruker, Germany). T2-weighted images were acquired using a multislice multiecho sequence: TE/TR 33 ms/2500 ms. Lesion sizes were quantified on these images using ImageJ (NIH software v1.45r, National Institute of Health, Bethesda, MD, USA). Two-dimensional time-of-flight angiographies (TE/TR 12 ms/7 ms) were acquired and analyses of the MCA angiogram were performed blinded to the experimental data using the following score: 1: complete obstruction, 2: partial occlusion with only 1 visible branch of the MCA, 3: partial occlusion with 2 or more terminal branches visible, 4: normal appearance of the MCA.

**Histological analyses**

Mice were euthanized 24h after stroke. The brains were removed and frozen in isopentane. Cryostat-cut coronal brain sections (20 µm) were stained with thionine. For volume analysis, 1 section out of every 10 was stained and analyzed using ImageJ (National Institute of Health). The lesion volume was estimated as the sum of every lesion area (unstained region) for each section, multiplied by the distance between each section (0.2 mm). Although the ischemic lesions defined by DWI and histology are closely related, we cannot exclude an effect of the tissue processing method on the apparent lesion size measured by histology.

**Labeling of tPA and albumin with Alexa555 and Alexa680**

Actilyse and albumin were dialysed overnight at 4°C to remove arginine. Then, purified tPA was mixed with the N-succinimidyl ester of Alexa555 or Alexa680 (Invitrogen) for 4 h at 4°C with continuous stirring. The resulting solution was dialyzed in bicarbonate buffer overnight at 4°C to remove unbound dyes. Conjugates were frozen and stored at –80°C until further use.

**Immunohistochemistry**

Deeply anesthetized mice were transcardially perfused with cold heparinized saline (15 mL) followed by 150 mL of fixative (PBS 0.1 M, pH 7.4 containing 2% paraformaldehyde and 0.2% picric acid). Brains and livers were post-fixed (18 hours; 4°C) and cryoprotected (sucrose 20% in veronal buffer; 24 hours; 4°C) before freezing in Tissue-Tek (Miles Scientific, Naperville, IL, USA). Cryomicrotome-cut sections (10 µm) were collected on poly-lysine slides and stored at –80°C before processing. Sections were co-incubated overnight with rabbit anti-LRP-1 (1:1000; kind gift from Dr. D. Strickland), in veronal buffer (pH 7.4). Primary antibodies were revealed using Fab’2 fragments of Donkey anti-rabbit IgG linked to FITC (1:500, Jackson ImmunoResearch, West Grove, USA). Washed sections were coverslipped with antifade medium containing DAPI and images were digitally captured using a Leica DM6000 microscope-coupled coolsnap camera and visualized with Metavue 5.0 software (Molecular Devices, USA) and further processed using ImageJ 1.45r software (NIH).

**Two-photon microscopy**

Mice were anesthetized with a mixture of ketamine/xylazine (100/20 mg/kg). Then, mice were intubated and mechanically ventilated with N2O/O2 in order to limit respiratory movements during images acquisition. Mice were placed on the back and the liver was exposed by a midline abdominal incision. Then, a glass cover slip was positioned on the liver and a drop of water was used to allow immersion of a 25x objective. We administered FITC-
Dextran (70kDa, 300µg/mouse, Sigma-Aldrich) and Alexa<sup>555</sup>-tPA (2 mg/kg) through the tail vein. Fluorescent images were longitudinally acquired (5 min interval) using a Leica TCS SP5 MP microscope at 840 nm two-photon excitation (in plane resolution of 0.2x0.2 µm).

**Near-Infrared Fluorescence Imaging (NIRF)**

Alexa<sup>680</sup>-labeled tPA (160 µg per mouse) (Life Technologies, CA) or Alexa<sup>680</sup>-albumin (160 µg per mouse) (Life Technologies, CA) were intravenously injected to control and alcohol-exposed mice. After 90 min, brains and livers (n=3-5 per group) were harvested and placed in the planar NIRF system (Photon Imager; Biospace). In order to bypass the requirement for normalization, measurement of the fluorescence of all the brains was performed at the same time, and the same method was used for the livers. Therefore, background fluorescence was the same for all organs. Excitation wavelength was set to 650 nm with a 700-nm high-pass emission filter. Autofluorescence images were acquired using an excitation wavelength of 590 nm with the same emission filter. Fluorescence was quantified on the whole brain or liver lobe using M3Vision software (Biospace).

**Blood-Brain Barrier (BBB) permeability measurements.**

BBB impairment was investigated using Evans blue extravasation. Evans blue (50µl of 4% solution ; Sigma) was injected in the tail vein 22 hours after stroke. After 2 hours, mice were deeply anesthetized and were transcardially perfused with cold heparinized saline (15 mL). Brain were harvested and placed in the planar NIRF system (Photon Imager; Biospace). Excitation wavelength was set to 540 nm with a 660 nm high-pass emission filter. Fluorescence was quantified on the whole brain using M3Vision software (Biospace).

**tPA<sup>555</sup> plasmatic clearance measurements**

Alexa<sup>555</sup>-labelled tPA (Life Technologies, CA) (0.5 mg/kg) was injected in the tail vein and blood samples were taken at different times (0, 5, 15, 30 and 60 minutes post-injection) by an arterial femoral catheter. Plasmas were submitted to electrophoresis under reducing conditions in SDS 8% polyacrylamide gels and fluorescence was visualized with a camera (LAS400, ImageQuant).

**Immunoblotting**

Ice-cold TNT buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 0.5% Triton X-100)-dissociated tissues were centrifuged (12,000 g, 4°C, 15 min), and protein content assessed by the BCA method (Pierce, France). Proteins (20 µg) were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with TBS (10mM Tris; 200mM NaCl; pH 7.4) containing 0.05% Tween-20, 5% BSA, and incubated overnight at 4°C with antibody against β chain of LRP-1 (85 kDa) (1 µg/mL) (Abcam). After incubation with the anti rabbit peroxydase-conjugated secondary antibodies (1:50000), proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system (Perkin Elmer-NEN, France).

**Quantitative real-time PCR**

Total RNAs were extracted from cultured cells by using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. Total RNAs (1 µg) from each sample were reverse-transcribed using the iScript Select cDNA Synthesis Kit (Bio-Rad). Primers were designed for each gene using the Beacon Designer software (Bio-Rad). Primer alignments were performed with the BLAST database to ensure the specificity of primers. PCR reagents were prepared with RNase-free water-containing primers and IQ SYBR Green Supermix (Biorad). PCR amplification was performed as described elsewhere (Rubio et al.,
pPIB was used as housekeeping genes for liver and brain samples. The levels of expression of gene of interest were computed as follows: relative mRNA expression = \( E^{-\Delta \text{Ct of gene of interest}}/ E^{-\Delta \text{Ct of housekeeping gene}} \), where Ct is the threshold cycle value and E is efficiency.

Hydrodynamic transfections
The full length human tPA cDNA was subcloned into the eukaryotic expression plasmid pLIVE® between NheI and BamHI. The construct was then amplified in Escherichia coli DH5α cells and purified by a Nucleobond endotoxin-free plasmid DNA PC 2000 kit (Macherey-Nagel) according to the manufacturer's instructions. The purity and quantity of the plasmids DNA were analyzed by absorbance at 260 and 280 nm.

C57BL/6 mice were injected with 100 µg of the pLIVE-vector containing or not the cDNA encoding for wild-type murine tPA (pLIVE-tPA) in saline (volume corresponding to 10% of body weight), as described by supplemental reference 2. DNA injection in the tail vein was completed in less than 5 s and the level of tPA expression in the liver and plasma was assessed 24h, 48h and 72h after injection of plasmid DNA. A negligible mortality was observed during this procedure (<3%).

Monitoring of Cerebral Blood Flow
Cerebral blood flow (CBF) was continuously measured by laser Doppler flowmetry using an optic fiber probe (Oxford Optronix) affixed on the skull above the MCA downstream of the injection site. CBF was measured before the injection (100% baseline) and throughout the duration of the experiment (60 minutes). The post-ischemic CBF was expressed as the percentage of CBF at two time points: at clot formation and at the end of the tPA or saline infusion over the baseline CBF.

Endogenous and exogenous tPA levels measurements

Amidolytic activity assay
Plasmas (without or with an i.v. injection of tPA at 10 mg/kg) were incubated in the presence of a fluorogenic substrate (5 µM) (Spectrofluor 444FL). The reaction was carried out at 25 °C in 50 mM Tris (pH 8.0) containing 150 mM NaCl in a total volume of 100 µl. The amidolytic activity of tPA was measured as the change in fluorescence emission at 440 nm (excitation at 360 nm).

Fibrin agarose zymography
Proteins from plasma samples (20 µg) and tPA as standard protein (10 µl, 0.06 IU/ml) were subjected to SDS electrophoresis (8% polyacrylamide gel under non-reducing conditions). SDS was then exchanged with 2.5% Triton X-100. After washing-out excess of Triton X-100 with distilled water, gels were carefully overlaid on a 1% agarose gel containing 1 mg/ml of bovine fibrinogen containing plasminogen. Zymograms were allowed to develop at 37 °C during 12 h and photographed at regular intervals using dark-ground illumination. Active proteins in plasma samples were identified by reference to the migration of known markers (tPA).

ELISA analysis
Both ELISA for murine active tPA and total antigen (Free, latent, and complexed) PAI-1 were performed on murin citrated plasma samples, according to the manufacturer’s instructions (Molecular Innovations®, USA).
Plasma clot lysis assay
The time to clot formation and the ability of tPA to lysate clot were studied in control and alcohol-exposed mice plasmas, by monitoring the change in turbidity. To initiate the clot formation, 25 mM calcium chloride was added to citrated plasma (25µl) diluted 1:2 in HEPES buffer [10 mM HEPES (pH=7.4), NaCl 150 mM, 0.4% BSA, 0.01% Tween 20]. After mixing, samples were incubated at 37°C and optical density was measured (405nm and 490) during 4 hours every 30 seconds. After the addition of 10nM of tPA, the rate of clot lysis was calculated as the time from initiation of clot formation to the time at which maximal absorbance falls to 50%. Tests were performed in triplicate. Results were expressed as the time to clot formation and the time to 50% clot lysis with tPA, in minutes.

Haemostatic parameters
Blood was collected by intracardiac puncture and stored in pre-citrated tubes (10% citrate buffer 0.129M). Platelet poor plasmas were prepared by a first centrifugation at 1500 rpm (15 min) followed by a 2 min centrifugation at 12000 rpm. Prothrombin time (PT), factor VIII and factor V were automatically measured on a ACL-Top coagulation analyzer (IL) using RecombiPlastin (IL) for PT, Factor VIII deficient plasma (Siemens) and SynthasIL (IL) for factor VIII, Factor V deficient plasma (Stago) for factor V and normal human plasma (ORKL, Siemens) used as a standard. Hematocrit was calculated as the percentage of red blood cells in blood.

Platelet count
Peripheral blood platelets were counted on a haemocytometer by a blind experimenter using a bright field microscope after red blood cell lysis, following the instructions of the manufacturer (Thrombo-TIC, Subra).

Statistical analyses
Results are the mean ± SEM. Statistical analyses were performed by the ANOVA test followed by post hoc comparison with the Fisher test (when comparing more than 2 experimental groups), or the t-Student test (when comparing 2 experimental groups), using the Statview software.
Supplemental table I: Physiological and haemostatic parameters in control and alcohol-exposed mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>40.0 ± 0.9</td>
<td>39.4 ± 0.7</td>
</tr>
<tr>
<td>Brain weight (mg)</td>
<td>477.5 ± 8.4</td>
<td>461.0 ± 5.5</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>2.19 ± 0.1</td>
<td>2.24 ± 0.1</td>
</tr>
<tr>
<td>Relative brain weight (%)</td>
<td>1.19 ± 0.03</td>
<td>1.17 ± 0.02</td>
</tr>
<tr>
<td>Relative liver weight (%)</td>
<td>5.47 ± 0.13</td>
<td>5.68 ± 0.12</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47.2 ± 1.40</td>
<td>50.4 ± 0.91</td>
</tr>
<tr>
<td>Prothrombin time (%)</td>
<td>135.3 ± 5.9</td>
<td>91.0 ± 8.9 ***</td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>27.4 ± 1.0</td>
<td>26.7 ± 1.0</td>
</tr>
<tr>
<td>Factor V (UI/ml)</td>
<td>2.05 ± 0.06</td>
<td>1.52 ± 0.19 *</td>
</tr>
<tr>
<td>Factor VII (UI/ml)</td>
<td>1.35 ± 0.08</td>
<td>1.22 ± 0.05</td>
</tr>
<tr>
<td>Factor VIII (UI/ml)</td>
<td>0.32 ± 0.03</td>
<td>0.53 ± 0.04 **</td>
</tr>
<tr>
<td>Antithrombin (UI/ml)</td>
<td>1.07 ± 0.03</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>Platelet number (x107)</td>
<td>227 ± 18</td>
<td>149 ± 7 ***</td>
</tr>
</tbody>
</table>

aPTT, activated partial thromboplastin time. n=6 mice/group; * p<0.05, ** p<0.01, *** p<0.001.
Supplemental figure I

![Graph showing comparison of CBF (per cent) between control and alcohol groups at 4h and 24h post-ischemia.](image)
Supplemental figure II
Supplemental Figure III

A. Monitoring of Cerebral Blood Flow (Doppler flowmetry)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cerebral Blood Flow (% of baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>120</td>
</tr>
<tr>
<td>Alcohol + tPA</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>80</td>
</tr>
<tr>
<td>Control + tPA</td>
<td>60</td>
</tr>
</tbody>
</table>

B. Angiographic score 2h30 after ischemic onset (MRI)

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>tPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.67 ± 0.33</td>
<td>3.50 ± 0.50</td>
</tr>
<tr>
<td>Alcohol</td>
<td>2.40 ± 0.40</td>
<td>3.00 ± 0.58</td>
</tr>
</tbody>
</table>

Score 1-4 (1=complete obstruction; 4=complete reperfusion)

C. Plasma clot lysis assay

- Control
- Control + tPA
- Alcohol
- Alcohol + tPA

Optical density (405/480 ratio) vs. Time (min)

D. 75% Clotting Time

- Control
- Alcohol

E. Half-lysis time (+tPA)

- Control
- Alcohol

F. Endogenous tPA levels (ELISA)

- Control
- Alcohol

G. Endogenous tPA activity assay

- Control
- Alcohol
Supplemental figure IV

A

![Graph A showing total PAI-1 plasma levels](image)

B

![Graph B showing liver PAI-1 mRNA levels](image)
Supplementary figure V

A

Liver mannose receptor mRNA levels (% vs. Control)

Control   | Alcohol
0          | 120
20         | 140
40         | 160

B

Brain mannose receptor mRNA levels (% vs. control)

Control   | Alcohol
0          | 100
20         | 120
40         | 140

ns
Figure Legends

Supplemental figure I: Effect of alcohol on cerebral perfusion 4h and 24h after stroke onset. Cerebral blood flow (CBF, %) of ipsi- vs. contralateral ROI was measured by Speckle contrast imager at two different times post-ischemic stroke onset, showing no differences on cerebral perfusion between alcohol and control group. ns=non significant differences were observed between groups.

Supplemental figure II. Effect of alcohol consumption on BBB integrity following stroke, measured by Evans blue extravasation. Evans blue fluorescence shows that alcohol do not alters the extend of BBB leakage following ischemic stroke neither in the saline nor the thrombolysed (tPA) group. (n=3-4 animals per group).

Supplemental figure III. No changes in fibrinolytic efficiency of exogenous tPA, endogenous tPA levels or activity. A, Monitoring of cerebral blood flow registered during surgical approach by Doppler flowmetry (n=7-8 mice/group). No differences in doppler reduction after thrombin injection (clot formation) or tPA-induced recanalization (doppler register at the end of saline/tPA injection) were noticed between control and alcohol group. B, IRM angiographic score 2h30 after stroke onset (1= complete obstruction; 4= complete recanalization) (n=7-8 mice/group). C, Plasma clot lysis assay curves measuring the clotting time and the time to clot lysis after the addition of tPA, showing no significant differences between control and alcohol-exposed (n=8 mice/group). D, Quantification of 75% clotting time after the addition of tPA (n=8 mice/group). E, Quantification of half-lysis time after the addition of tPA (n=8 mice/group). F, Endogenous free-tPA levels measured by ELISA (n=8 mice/group; ns, not significant). G, Amidolytic activity assay of endogenous tPA (n=4 mice/group).

Supplemental figure IV. Effect of 6 weeks of alcohol exposure on PAI-1 expression Results are expressed as the mean ± SD. (A) Total (free, latent, and complexed) plasmatic PAI-1 levels in control versus alcohol-treated animals, measured by ELISA (n=10/group), showing that total PAI-1 levels are reduced in the plasma of the alcohol-exposed group, as compared to controls. (B) PAI-1 mRNA expression levels in the liver after 6 weeks of alcohol consumption, showing that alcohol reduces PAI-1 mRNA in the liver as compared to the control group (n=4/group). All experiments were performed in duplicate. Results are expressed as the fold-change relative to a housekeeping gene (GAPDH) *p<0.05 vs control.

Supplemental figure V: Effect of 6 weeks of alcohol consumption on mannose receptor expression. Results are expressed as the mean ± SD. Mannose receptor mRNA expression levels in the liver (A) and brain (B) after 6 weeks of alcohol consumption, showing that alcohol does not modify significantly Mannose receptor mRNA levels neither in the liver nor in the brain as compared to the control group (n=4/group). All experiments were performed in duplicate. Results are expressed as the fold-change relative to a housekeeping gene (GAPDH). ns= non significant differences were observed.
Supplemental references
