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REQUIREMENT OF ARGININOSUCCINATE LYASE FOR SYSTEMIC NITRIC OXIDE PRODUCTION

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Author contribution

A.E.- Generated the mouse models, performed most of the experiments and wrote the manuscript
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O.A.S.- Conducted the mouse therapy experiments
M.H.P.- Conducted the immunopercipitation experiments
P.M.C.- Helped conducting the mutated ASL experiments
Y.C.- Generated the mouse models
H. K.G.- Analyzed the NOx data and helped with the ELISA experiments
L.L.- Performed western experiments
A.M.- Conducted the patients' studies
T.K.B.- Conducted the RT-PCR experiments
J.O.B.- Performed the histological analysis
H.Z.- Performed western and immunopercipitation experiments.
Y.T.- Conducted the vascular ring experiments
A.K.R.- Helped with the blood pressure analysis
M.S.- Contributed to the conception of the hypothesis
W.E.O.- Analyzed the biochemical data
D.G.H.- Contributed to our understanding regarding NOS function
W.E.M.- Conducted the vessel reactivity assay, helped with assessing creatinine clearance
J.C.M.- Performed the labeled isotope studies
J.L.A.- Supervised the experiments performed on the Asl hypomorphic lungs, helped in critical analysis and in revising of the manuscript
N.S.B. – Helped with the NOx and NOS data analysis and supervised related experiments
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Abstract

Nitric Oxide (NO) plays a critical role in diverse physiological and pathological processes. We show that a hypomorphic mouse model of argininosuccinate lyase (*Asl*) deficiency exhibits a distinct phenotype manifest by multi-organ dysfunction and NO deficiency. Loss of Asl leads to reduced NO synthesis due to decreased endogenous arginine synthesis as well as reduced utilization of extracellular arginine for NO production in both humans and mice. Hence, ASL as seen in other species through evolution has a structural function in addition to its catalytic activity. Importantly, therapy with nitrite rescued the tissue autonomous NO deficiency in hypomorphic *Asl* mice, while a NOS independent NO donor restored NO-dependent vascular reactivity in subjects with ASL deficiency. Our data demonstrate a previously unappreciated role for ASL in NOS function and NO homeostasis. Hence, ASL may serve as a target for manipulating NO production in experimental models, as well as treatment of NO-related diseases.

L-Arginine is the natural substrate of nitric oxide synthases (NOS) for generating nitric oxide (NO). As a by-product of the NOS reaction, L-citrulline is formed from L-arginine. Within the cell, citrulline can be recycled back to arginine by the cytoplasmic enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), constituting the citrulline-NO cycle (Supplementary Fig. 1). The availability of intracellular arginine is potentially a rate-limiting factor in cellular NO production in spite of the fact that the extracellular and exogenous sources should theoretically be able to replenish its deficiency. It has been hypothesized that compartmentalization and intracellular metabolite channeling underlies the “arginine paradox” in which extracellular and intracellular pools of arginine are distinguishable. However, this has yet to be proven *in vivo* and the mechanistic basis is unknown.

Many tissues and cell types contain the cytoplasmic enzymes, ASS and ASL, providing a cell-autonomous mechanism for generating arginine (Supplementary Fig. 1). Arginine serves as the precursor for the synthesis of urea, NO, polyamines, proline, glutamate, creatine and agmatine[1]. The regulation of L-arginine availability for these intracellular pathways is cell-type and end-product specific, e.g., arginine generated by ASL in hepatocytes is mostly directed to urea production, while in other cell types its metabolic fate is context dependent[2].

Argininosuccinic aciduria (ASA) (MIM 207900) is the second most common human urea cycle disorder (UCD) and is caused by deficiency of ASL. Subjects with ASA disease cannot generate arginine from citrulline. It is noteworthy that despite early treatment and adequate metabolic control of hyperammonemia, subjects with ASA disease can exhibit persistent intellectual impairment, delayed motor skills[3,4] and progressive hepatic disease[5–7]. Importantly, intellectual impairment and liver cirrhosis are seen even in those with early initiation of treatment and few, if any documented episodes of hyperammonemia. Recent experience also suggests that patients are at risk for the development of systemic hypertension[4,8,9]. The mechanism behind these unique clinical features that are not observed in other UCDs is open to speculation, although a reduction of NO production secondary to a localized deficiency of L-arginine is one intriguing possibility. An association between urea cycle function and NO production has been previously suggested by the association of genetic variants in urea cycle genes with NO-related disease processes[10,11]. Recently, NO synthesis was evaluated in patients with UCD[12]. However, no mechanism was demonstrated that could explain potential differences amongst or between patients and controls. Such a study emphasizes the difficulties in studying NO metabolism at the level of the whole organism in non-steady state conditions.

We hypothesized that a cell autonomous deficiency of ASL would lead to systemic NO deficiency. In addition, because of the inability of supplemental arginine to prevent long-term complications in ASA patients, we investigated whether ASL plays a more central role in cellular arginine utilization for NO synthesis beyond intracellular recycling of citrulline into arginine. We tested our hypothesis *in vivo* in a hypomorphic mouse model of *Asl* as well as in ASA subjects with absent enzyme activity. Our results were complemented with *in vitro* studies in human ASA fibroblasts, in primary cell lines with ASL knockdown, and in cells over expressing ASL mutants that are enzymatically inactive but structurally intact.

RESULTS

Asl hypomorphic mice show evidence of multi-organ dysfunction

We hypothesized that loss of *Asl* would cause deficiency of endogenous arginine production resulting in reduced NO synthesis. Similar to human neonates with ASA, complete loss of function of *Asl* in mice leads to neonatal hyperammonemia and lethality[13]. Therefore, we generated a conditional hypomorphic allele by introducing a Neomycin (*Neo)* selection cassette into intron 9 of the mouse *Asl* gene (Supplementary Fig. 2a–c). As predicted, quantitative RT-PCR, Western blot analysis and Asl enzymatic activity of mice homozygous for the *Neo* insertion, $Asl^{Neo/Neo}$, demonstrated significant reduction in gene and protein expression with 25% residual RNA, 25% residual protein and 16% residual enzyme activity, thus confirming the hypomorphic nature of this allele (Supplementary Fig. 3a–c). Biochemically, plasma amino acid analysis also showed a profile consistent with Asl deficiency with elevation of citrulline and argininosuccinic acid, two precursor metabolites upstream of the enzymatic block, and reduction of arginine, a product metabolite downstream of the enzymatic block (Supplementary Fig. 3d). The hypomorphic nature of this allele was confirmed to be due to the *Neo* cassette insertion, as its Frt-mediated deletion resulted in phenotypically normal mice ($Asl^{Flox/Flox}$) (Supplementary Fig. 4a).

Asl hypomorphic mice were born at the expected Mendelian ratio but die within the first 3–4 weeks of life from multi-organ failure (Fig. 1a,b), providing an opportunity to explore the consequences of impaired endogenous arginine synthesis. During the early postnatal period, the dietary supply of arginine (from breast milk) is insufficient and thus, there is dependency on endogenous arginine synthesis[14].

While $Asl^{Neo/Neo}$ mice were indistinguishable from wild type littermates at birth, they showed abnormal hair patterning and significant growth restriction by two weeks (Fig 1a,c). $Asl^{Neo/Neo}$ mice also displayed evidence of multi-organ dysfunction characterized by elevation of liver transaminases, decreased renal creatinine clearance, and elevated systolic and diastolic blood pressures as compared to WT animals (Fig. 1d–f). Histological analyses revealed multi organ involvement, including the immune, hematopoietic, renal and cardiovascular systems, features that are not attributable to hyperammonemia[15] (Supplementary Figure 5a–f).

We noted that some features of this phenotype would be consistent with a systemic disruption of NO homeostasis, since NO has been shown to play an important role in diverse pathological processes including systemic hypertension, immune dysfunction, renal disease and hepatic fibrosis[16,17].

Loss of Asl leads to global NO deficiency in mice

To determine whether the physiological features observed in the *Asl* hypomorphic mice were attributable to impaired NO synthesis or altered NO homeostasis, we examined the effect of a genetic interaction between *Asl* and *Nos3* on systemic blood pressure. While

neither the *Nos3*$^{+/-}$ or the *Asl*$^{Neo/+}$ mice are hypertensive, the double heterozygous mice, *Nos3*$^{+/-}$;*Asl*$^{Neo/+}$ had significant hypertension (Figure 1g) confirming an epistatic effect.

While measurement of NO oxidative products (nitrite, nitrate) can reflect the status of NO production, we additionally assayed the tissue and plasma nitrosothiols (RSNO) levels as a reflection of NO production and signaling. We found reduced NO production in tissues of *Asl*$^{Neo/Neo}$ mice as reflected by a significant decrease in S-nitrosylation and/or nitrite in heart, and other tissues (Fig. 2a,b). To address whether the loss of Asl led to secondary changes within the NO pathway that might alternatively account for reduced NO synthesis, we measured RNA and protein expression levels of *Ass* (upstream of *Asl*), arginine transporter *Cat-1*, and *Nos3*. We found that their expression in mutant lung and liver was either unchanged or even up-regulated in the face of *Asl* loss of function (Fig. 2c, Supplementary Fig. 6a–c). Similarly, the levels of the endogenous inhibitor of L-arginine, asymmetric dimethyl arginine (ADMA), were also comparable in mutant and WT mice (**Data not shown**).

Hence, the multi-organ dysfunction observed in *Asl* mutant mice correlated with evidence of decreased systemic NO resulting specifically from the primary deficiency of Asl activity. This specificity was further supported by the finding of normal tissue RSNO levels in the *Asl*$^{Flox/Flox}$ mice in which the *Neo* cassette had been excised and Asl expression normalized (Supplementary Fig. 4b,c).

Treatment with alternative NO sources partially rescues *Asl* hypomorphic mice

Recent studies show that nitrate and nitrite can be recycled to form NO as an alternative to the classical L-arginine-NOS pathway[18,19]. If some of the phenotypic consequences of Asl deficiency were due to decreased NO production, normalization of NO status by supplementation with sodium nitrite should partially correct the phenotype. However, in this model, hyperammonemia (secondary to an inability to clear waste nitrogen by urea synthesis in the liver) complicates the postnatal picture and contributes to the lethality in *Asl*$^{Neo/Neo}$ mice. Therefore, we first treated *Asl* mutant animals with either sodium benzoate (250 mg kg^{-1} per day) or with L-arginine (100 mg kg^{-1} per day). These medications are the standard of care in ASA patients for prevention of hyperammonemia. The former works by stimulating alternative disposal of glycine containing nitrogen via conjugation, and the latter works by priming the urea cycle to generate more ASA as a nitrogen sink that is cleared via urinary excretion. Not surprisingly, we found significantly improved survival in *Asl*$^{Neo/Neo}$ mutant mice treated with these drugs suggesting that, similar to human patients, hyperammonemia is indeed partially responsible for early lethality in this model (Fig. 3a). Survival improved more with arginine treatment than sodium benzoate treatment suggesting that exogenous arginine may also have a salutary effect on NO deficiency state in addition to its effects on nitrogen clearance. However, the predominant contribution of NO deficiency to the phenotype was best evidenced by the observation that treatment with sodium nitrite, which can be metabolized to form NO[18,19], produced the greatest survival among all treated groups (Fig. 3a, Supplementary Table 2). Nitrite supplementation produced comparable survival to the traditional treatment of sodium benzoate plus arginine; however, weight gain was only evident in the nitrite group (Fig. 3b,c). Importantly, combination triple therapy with sodium benzoate, arginine, and sodium nitrite, produced the greatest survival and weight gain (Fig. 3b,c). Moreover, triple therapy was associated with correction of liver protein nitrosylation to levels higher than those observed in the WT mice and led to normalization of the blood pressures in mutant mice (Fig. 3d,e).

Tissue and cellular NO deficiency despite adequate arginine treatment

To evaluate for NO insufficiency at a tissue level in $Asl^{Neo/Neo}$ mice, we performed classic aortic ring relaxation measurements. In contrast to WT mice, aorta from $Asl^{Neo/Neo}$ mice exhibited evidence of significant endothelial dysfunction as shown by an inability of preconstricted aortic rings to relax in response to acetylcholine (Fig. 3f,g). Furthermore, neither preconstricted aortic rings from WT nor $Asl^{Neo/Neo}$ mice responded to arginine treatment. A lack of response in WT rings *ex vivo* was expected as the vascular tissues are saturated with arginine to levels higher than the K_m values of NOS for NO production[20–22]. However, this result was surprising in the $Asl^{Neo/Neo}$ mouse aortas given their low intracellular levels of arginine. In contrast, $Asl^{Neo/Neo}$ preconstricted aortic rings relaxed in response to sodium nitroprusside, a NOS independent NO donor, demonstrating the integrity of the vascular preparation and a functional signaling pathway downstream of NO (Fig. 3g).

To further evaluate the requirement of Asl for NO synthesis in a cell specific manner, we knocked down its expression using RNAi in primary piglet endothelial cells (Figure 4a,b). We found that decreased levels of ASL led to significantly lower levels of nitrite production in response to inducers such as bradykinin and L-arginine (Figure 4c,d).

Together, these data underscore the requirement for Asl in the production of NO at the organism, tissue, and cellular levels.

ASL mutant cells and ASA patients have deficiency of NOS-dependent NO production

Our findings from the hypomorphic mouse model and *in vitro* studies predict that human ASA patients should also be deficient for markers of NO synthesis in spite of normal or even elevated levels of extracellular plasma arginine from therapeutic arginine supplementation provided as “standard of care”. To assess the role of ASL in NO synthesis in humans, we studied fibroblasts from human subjects with ASA disease with null ASL enzymatic activity. First, we verified that primary human fibroblasts express both *NOS3* and *NOS2* (Supplementary Fig. 7a). Although the expression levels of both *NOS2* and *NOS3* are lower than those seen in endothelial cells, both isoforms were detected at comparable levels in cultured human fibroblasts (Supplementary Fig. 7a). In addition, as previously published[23–25], we established that NOS is functional in primary fibroblasts and that it can be activated by BH_4 and inhibited by a NOS inhibitor (Supplementary Fig. 7b). As predicted from our mouse and cell data, addition of either L-arginine (1000μM) or L-citrulline to the media of primary fibroblasts from control subjects for either 30 minutes or 24 hours resulted in significant increases in nitrite and cGMP production reflecting an increase in NO synthesis from NOS. In contrast, nitrite production, nitrosylation, and cGMP production in fibroblasts from subjects with ASA did not increase in response to L-arginine treatment despite similar expression of NO synthetic proteins (Fig. 5a,b and Supplementary Fig. 7c–f). The viability of cells from both controls and subjects with ASA disease cells was comparable and intracellular arginine levels were effectively increased in both cell types by supplementation (**Data not shown**) and yet, normal NO synthesis did not occur in the absence of ASL.

Deficiency of arginine utilization for NO synthesis in subjects with ASA

In spite of elevations in plasma arginine (as a consequence of therapeutic supplementation) and of citrulline (that accumulates upstream of the block), subjects with ASL deficiency had significantly decreased levels of plasma RSNO and decreased nitrite as compared to healthy control subjects (Fig. 5c). In order to directly measure NO production and trace the contribution of extracellular arginine to NO conversion, we performed stable isotopic flux measurements in control and ASA subjects on a steady state nitrite and protein restricted diet. Using a two-day multi-tracer protocol, we found increased arginine and urea

appearance (flux) rates in ASA subjects that are attributable to pharmacological treatment with L-arginine (Fig. 5d-**Left Panel**). We also found increased citrulline flux and fractional transfer of ^{15}N label from ^{15}N-glutamine to ^{15}N-citrulline that is explained by the accumulation of this metabolite upstream of the block in ASL deficiency. However, consistent with the decreased plasma RSNO levels and the mouse and cellular data, we found dramatically decreased ^{15}N transfer from infused $^{15}N_2$-guanidino-labelled-arginine to ^{15}N-citrulline, a surrogate marker for NO production (Fig. 5d-**Right Panel**). Hence, dynamic measurements of metabolite fluxes reveal that in spite of high extracellular arginine and citrulline fluxes, ASA subjects have dramatically decreased transfer of guanidino nitrogen from arginine to citrulline, a marker of NO production from arginine. Our data suggest that the decrease in NO production is attributable to an inability both to recycle intracellular citrulline into arginine for endogenous synthesis, and to channel extracellular plasma arginine for NO production. These complementary human data further support our finding, that in the absence of ASL, extracellular arginine (the source of the isotopic label) is inefficiently converted to citrulline to form NO. Hence, ASL deficiency is a model of deficient NO synthesis from both intracellular and extracellular sources of arginine.

To evaluate the functional consequences of this observation *in vivo*, we performed vessel reactivity assays in human subjects with ASA. We examined the vasodilatory response of the brachial artery via Doppler ultrasound following transient occlusion of flow as a measure of NOS-dependent vascular relaxation, and then the vasodilator response following sublingual nitroglycerin as a measure of NOS-independent vascular relaxation (Fig. 5e). In healthy controls, vascular dilatation following both steps were equivalent and the magnitude of response was similar to previously reported responses[26]. In contrast, subjects with ASA disease failed to show any NOS-dependent vascular relaxation after release of vessel occlusion. However, they responded similarly to controls after administration of sublingual nitroglycerin (Fig. 5e) showing normal response to an exogenous, NOS-independent source of NO. These results are significant as the near absence of flow-mediated relaxation, a measure of NO mediated dilation, has not been reported in other pediatric diseases. Importantly, these results in human subjects corroborate with the aortic ring experiments in the hypomorphic *Asl* mouse model and support that while the signaling downstream of NO is intact, loss of ASL limits NOS-dependent NO production, leading to loss of NOS-dependent vascular reactivity.

ASL is required for maintenance of a NOS multi-protein complex

Direct biochemical interaction within a compartmentalized NOS complex may explain metabolite channeling[27,28]. The three key protein components responsible for recycling citrulline into NO are ASS, ASL, and NOS and their expressions are coordinately regulated[29]. Several studies have shown either interaction or colocalization amongst these three proteins[30,33]. Interestingly, an interaction between NOS3 and the cationic amino acid transporter CAT-1 responsible for arginine transport, was also recently described in endothelial cells[30].

We first verified the interaction between the proteins involved in the complex by performing a mass spectrometry analysis of immunoprecipitate using an antibody to ASS on LPS induced RAW246.7 cells, which confirmed the existence of this complex for Nos2 (Supplementary Table 1). We expanded upon these findings by testing the existence of a complex between the protein components involved in NO synthesis by performing immunoprecipitation using antibody to ASS in lung and brain tissues from WT mice. Our results support the existence of a NOS-containing multi-protein complex for both NOS isoforms i.e., Nos3 in lung and Nos1 in brain (Fig. 6a, Supplementary Figure 8).

To determine the effects of partial loss of Asl on this protein complex, we quantified the component proteins co-immunoprecipitated by antibody to ASS in WT vs. $Asl^{Neo/Neo}$ mice. Since ASA patients have hypertension and intellectual delays that are unique and independent of hyperammonemic episodes, we focused our studies on the effect of Asl deficiency on its complex formation with Nos3 and Nos1. $Asl^{Neo/Neo}$ mouse lung and brain showed significantly decreased quantities of both Nos3 and Nos1 isoforms, respectively, as well as other proteins involved in the complex (Fig. 6a, Supplementary Fig.8); this is despite increased abundance of the individual components (other than Asl) in tissues of $Asl^{Neo/Neo}$ mice compared to WT on Western analysis (Figure 2c, Supplementary Fig.8). These data support a central requirement for Asl in the formation of this complex.

To dissect the structural requirement of ASL for NOS complex formation from its catalytic activity, we tested NOS complex assembly using human ASL mutants in its catalytic site R236W[31] (Fig. 6b). This mutation abolishes the enzymatic activity of ASL, i.e., the recycling of intracellular citrulline via the cleavage of ASA, but does not abolish its tertiary structure. Indeed, *in vitro* over-expression of mutated ASL in COS7 cells did not prevent NOS protein assembly with ASS as shown by IP using antibodies against ASS or NOS. However, consistent with our finding in the $Asl^{Neo/Neo}$ mouse, absence of ASL prevented efficient immunoprecipitation of the proteins involved in the NOS complex by either one of these antibodies (Fig. 6b).

To test whether this structural requirement for ASL in NOS complex formation is in fact required for utilization of extracellular arginine for NO production, we transduced ASA mutant fibroblasts null for ASL activity with lentivirus expressing either wild type human ASL or with human ASL mutated in the catalytic domain R113Q[31]. As extensively studied by others, the R113Q similarly abolishes the catalytic activity of ASL without affecting ASL protein stability[31,32]. If the structural requirement of this NOS complex is required for channeling of extracellular arginine to NOS, the catalytic site mutation would be expected to restore NO production in response to extracellular arginine in ASA mutant cells. Indeed, when we overexpressed human ASL with the R113Q mutation *in vitro*, ASA fibroblasts were able to generate nitrite at a level comparable to control cells when supplemented with arginine (Figure 6c).

This structural requirement for an ASL-dependent NOS complex begins to explain our physiological and cellular observations, suggesting that in the absence of ASL, there is a less efficient formation of a NOS multi-protein complex leading to decrease NO production from both endogenously synthesized and exogenously channeled arginine (Fig. 6d).

DISCUSSION

Our findings support a requirement for ASL not only to synthesize intracellular arginine, but also to utilize extracellular arginine for NOS-dependent NO synthesis. We demonstrate an intracytosolic complex of proteins important for nitric oxide synthesis to explain the structural basis for metabolite channeling that reconciles the phenotypes observed in human and mouse models of ASL deficiency. While likely not the only explanation, the data support that decreased ASL levels leads to loss of NOS complex formation that is associated with NO deficiency at the whole organism, tissue and cellular levels in both humans and mice.

This conclusion is based on a combination of mouse and human studies that were prompted by clinical observations in ASA patients. First, the natural history of ASL-deficient humans with argininosuccinic aciduria suggested the presence of organ dysfunction and complications that were independent of hyperammonemia caused by hepatic urea cycle

deficiency. This led us to hypothesize alternative mechanisms of injury including deficiency of NO secondary to loss of citrulline recycling or endogenous arginine production. However, this could not alone explain the phenotypic complexity because ASL patients are replete with extracellular arginine due to pharmacological supplementation.

This apparent conundrum was evident in the hypomorphic mouse model of Asl deficiency, where we observed histological evidence of multi-organ dysfunction that correlated with biochemical evidence of systemic NO deficiency. This was further supported by the finding of decreased markers of NO production (plasma RSNO and nitrite) in ASA patients. Importantly, ASA patients, ASA fibroblasts, and primary endothelial cells made deficient of Asl by siRNA knockdown, were also unable to efficiently generate NO after extracellular arginine supplementation. In humans, this was demonstrated via dynamic measurement of arginine to citrulline flux; while in cells, it was demonstrated via measurement of nitrite and/or cGMP. This NO deficiency was found to be tissue autonomous as preconstricted aortic rings from mutant mice relaxed in response to NO donors but not arginine, while ASA subjects exhibited abnormal flow-mediated vascular relaxation that was restored by nitroglycerin. On the whole organism level, provision of an NOS independent source of NO in the form of nitrite therapy significantly prolonged survival of hypomorphic mice while also restoring tissue nitrosylation and normalizing blood pressure.

The NO deficiency was caused in part by the inability of patients and cells to efficiently generate intracellular arginine or to utilize extracellular arginine for NO production in the face of ASL deficiency. This was in spite of adequate expression of all other protein components necessary for NO production including the arginine transporter CAT-1, ASS, HSP90, and NOS. This observation correlated with the existence of a NOS complex that depends on the structural, but not enzymatic function of ASL. Loss of ASL was associated with decreased abundance of this complex, decreased utilization of arginine for NO production, and functional consequences of NO deficiency at the organ and cellular levels.

This structural requirement was supported by the ability of specific mutations in the ASL catalytic domain to participate in the NOS complex while the complete absence of ASL prevented efficient complex formation. Together, these studies distinguish two essential roles for ASL: the recycling of citrulline in the cell for cell autonomous arginine synthesis, and the maintenance of a NOS complex that is required for efficient NO production from extracellular sources of arginine. The former depends on the catalytic function of ASL, while the latter requires its structural integrity. Interestingly, this is consistent with the distinct evolutionary roles of ASL in other species[33]. Since in cell, mouse, and patient models, the NO deficiency are evidenced in the face of excess arginine, it is likely that the primary dysfunction is at the level of the NO metabolon where insufficient channeling of arginine due to loss of the ASL-NOS complex leads to secondary decreased NO production. An important question for future study is whether the loss of arginine channeling leads to NOS uncoupling and consequent increase in free radical stress.

Clinically, these data suggest that the vascular dysfunction and intellectual delay seen in ASA patients may be partly due to NO insufficiency, and hence, treatment with a NOS-independent source of NO, e.g., sodium nitrite, or NO donors, would be beneficial in the long term. Moreover, the clinical variability seen in ASA patients may depend on the differential effects of specific mutations on catalytic vs. structural functions.

These data also have broader implications for NO biology and disease. Mechanistically, they support intracellular compartmentalization as an explanation for the “arginine paradox”, i.e., the increased production of NO with the addition of extracellular arginine despite apparently saturating intracellular arginine levels. Moreover, they suggest an explanation as to why the

arginine paradox is not observed with ASL deficiency. As such, ASL may serve as the linchpin in NO production. Hence, inhibition of ASL in a cell-specific fashion may be an effective way to probe NO function *in vivo,* independent of potential NOS redundancy. Similarly, it may serve as a novel target for manipulating NO production in a cell autonomous fashion in human disease processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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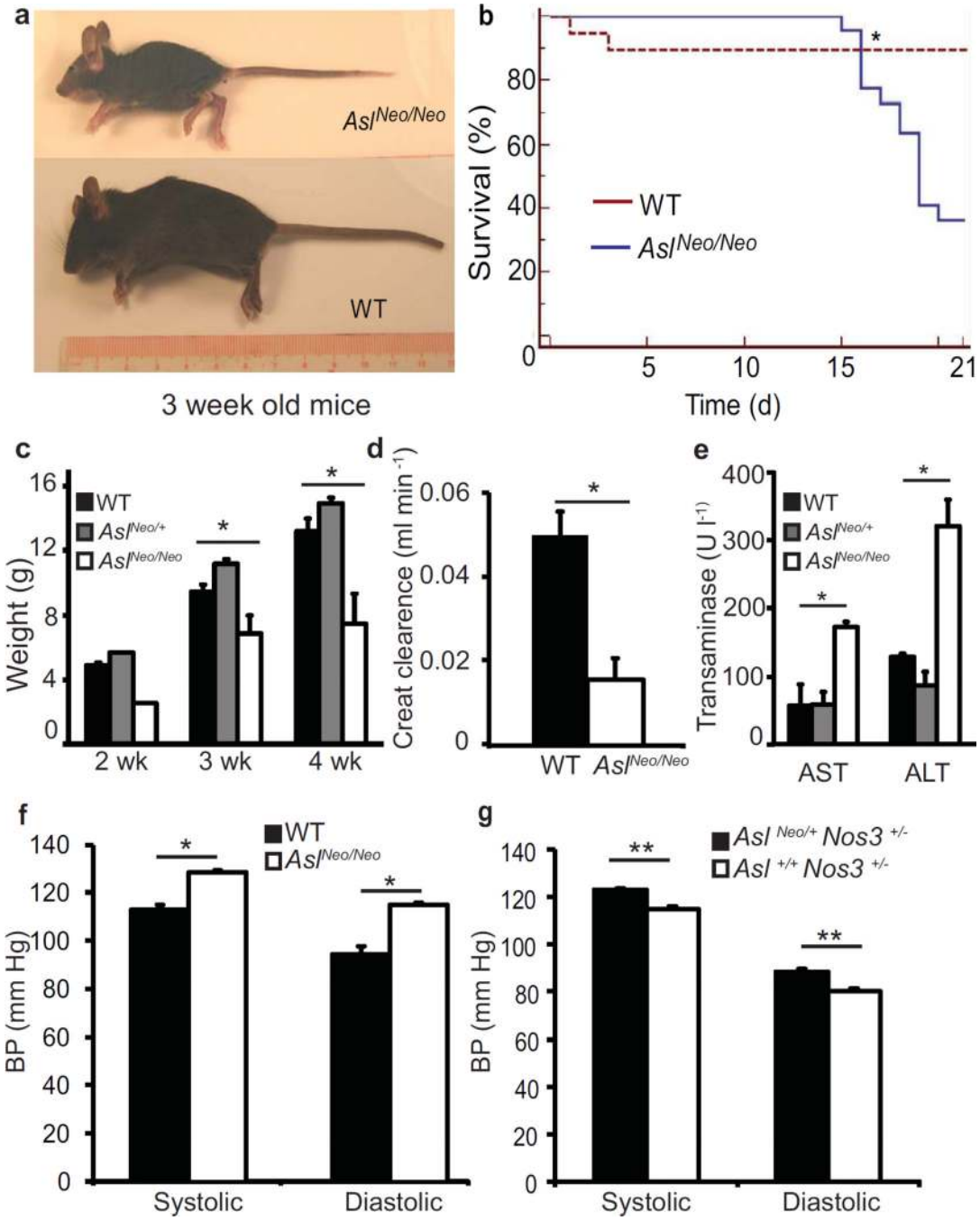

Figure 1. Physiological phenotype of *Asl*$^{Neo/Neo}$ mice

(**a**) Growth and hair phenotype. (**b**) Kaplan Meier survival curve for *Asl*$^{Neo/Neo}$ mice (n=22; solid line) vs. WT mice (n=19; dotted line) (*p<0.002). (**c**) Weight for *Asl*$^{Neo/Neo}$ mice (n=5) vs. WT (n=4) or heterozygous (n=6) littermates. WT: wild type; *Asl*$^{Neo/+}$: heterozygous mice; *Asl*$^{Neo/Neo}$: homozygous mice (*p<0.05). (**d**) Renal Function for *Asl*$^{Neo/Neo}$ mice (n=4) vs. WT littermates (n=5) (*p<0.005). (**e**) Liver transanimases for *Asl*$^{Neo/Neo}$ *mice* (*p<0.05). (**f**) Blood pressure measurements for *Asl*$^{Neo/Neo}$ mice (n=6) vs. WT littermates (n=4) (*p<0.0002). (**g**) Blood pressure in *Nos3*$^{+/-}$;*Asl*$^{Neo/+}$ (n=9) double heterozygous vs. *Nos3*$^{+/-}$;*Asl*$^{+/+}$ mice (n=4) (**p<2×10^{-8}).

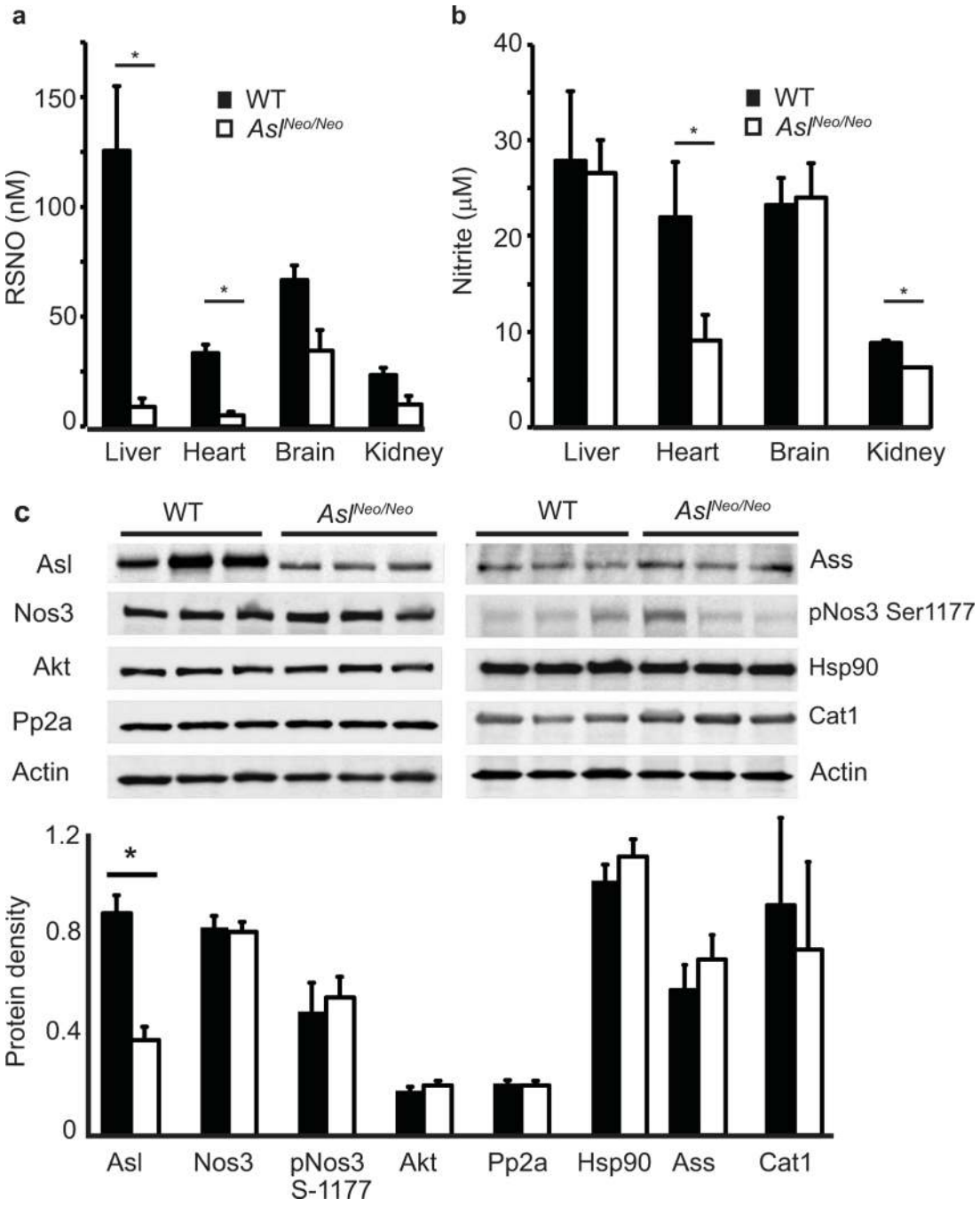

Figure 2. Decreased NO production with ASL deficiency

(**a,b**) Nitric oxide status in *Asl*$^{Neo/Neo}$ mice (n=3) and WT littermates (n=5). (**a**) Nitrosothiol measurements (RSNO) (*p<0.05). (**b**) Nitrite measurements (*p<0.05). (**c**) Representative Western Blot analyses of lung tissue samples from 10 *Asl*$^{Neo/Neo}$ mice and 10 WT control. Antibodies used are labeled on side. Protein densitometry is shown in the bottom panel.

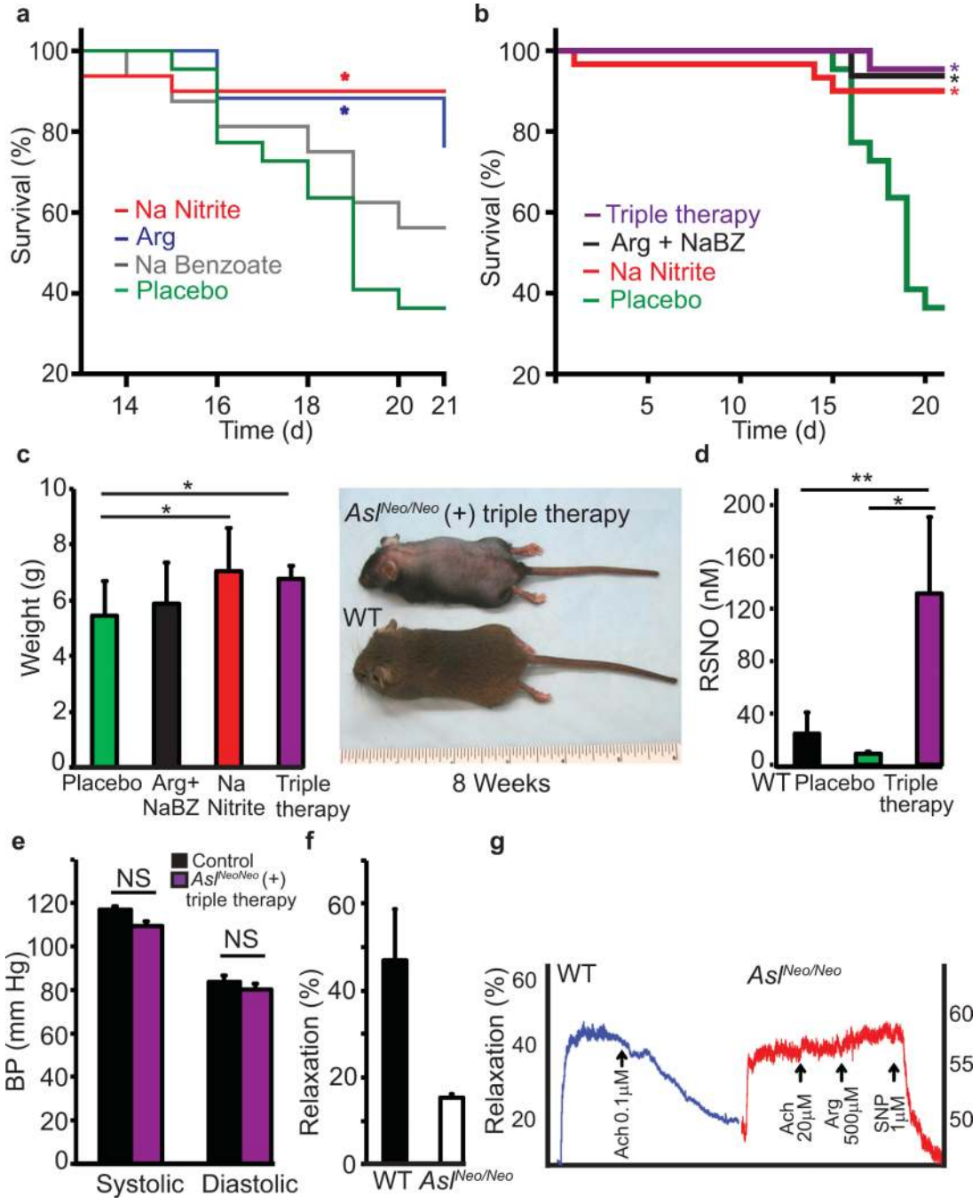

Figure 3. Pharmacological rescue with an NO source

(**a**) Kaplan Meier survival curves comparing different treatment modalities *vs.* placebo in *Asl*$^{Neo/Neo}$ mice (*p<0.007) (n = 15–22 in each group). (**b**) Kaplan-Meier survival analysis (*p<0.001 on log ranks) between the arginine plus sodium benzoate treated group (n=16), the sodium nitrite treated group (n=27) and the triple therapy group (n=22), when compared to the placebo group (n=22). (**c**) Left panel: Weight gain with sodium nitrite treatment (n=29) and the triple therapy treatment (n=22) as compared to placebo (n=26) and to the standard treatment with arginine and sodium benzoate (n=17) group (one-way ANOVA, *p <0.05). Right panel: A representative picture of WT control and *Asl*$^{Neo/Neo}$ mice at 8 weeks

of age after receiving the triple therapy. (**d**) Nitrosothiols (RSNO) were measured in liver of *Asl*$^{Neo/Neo}$ and WT mice treated with placebo vs. triple therapy (arginine, sodium nitrite and sodium benzoate) (*p<0.01; **p<0.009). (**e**) Blood pressure measurements in WT (n=3) vs. *Asl*$^{Neo/Neo}$ (n=4) on triple therapy with sodium benzoate, arginine, and sodium nitrite. (**f,g**) **Aortic ring relaxation.** (**f**) A representative graph for the difference in WT aortic rings relaxation vs. *Asl*$^{Neo/Neo}$ aortic rings relaxation (p<0.004). The graph represents the percentage relaxation in response to acetylcholine measured in 3 WT and 3 *Asl*$^{Neo/Neo}$ aortae, 4 segments from each aorta were analyzed (p<0.004). (**g**) Representative tracing from WT vs. *Asl*$^{Neo/Neo}$ aortic ring isometric tension studies showing percentage relaxation in response to acetylcholine (Ach), arginine (Arg), and sodium nitroprusside (SNP) treatment

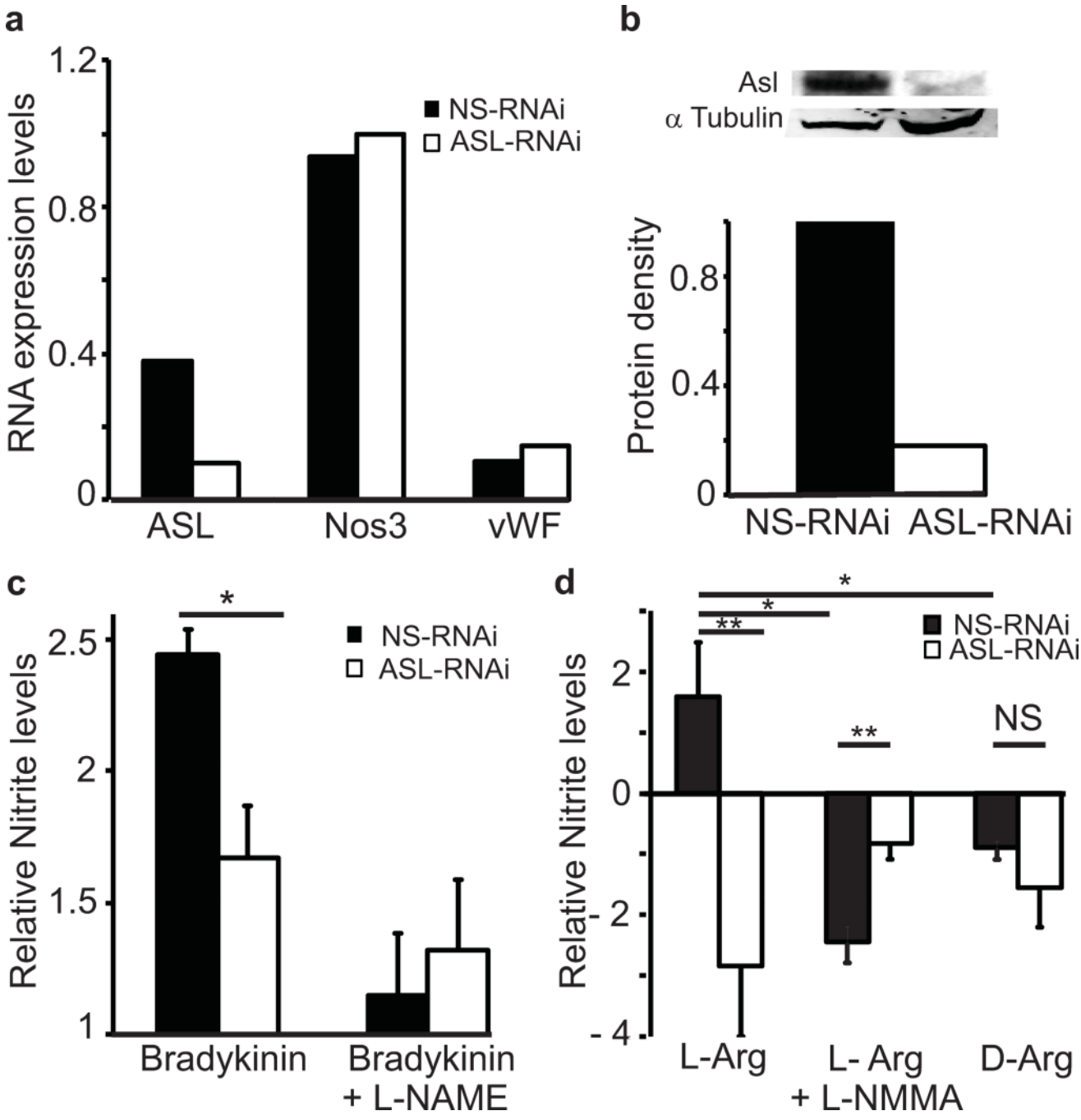

Figure 4. *Asl* knockdown in piglet endothelial cells decreases nitrite production in response to arginine

(**a**) Relative RNA expression in pooled RNA from primary piglet pulmonary artery (PA) endothelial cells transfected with Asl siRNA (ASL-RNAi) compared to non-specific siRNA (NS-RNAi). Endothelial specific von Willebrand factor (vWF) and NOS3 levels are shown as control. (**b**) Western blot using antibody to ASL in endothelial cells transfected with ASL siRNA as compared to NS-RNAi siRNA. The densitometric quantification is below. (**c**) Measurement of the relative nitrite levels in the media of piglet PA endothelial cells transfected with ASL siRNA vs. NS siRNA in response to Bradykinin (10 μM) treatment (for 24 hours) with and without the NOS inhibitor L-NAME (100 μM) (*p<0.05). The

experiment was performed in triplicate. Please refer to the online methods for the specific calculation (**d**) Measurement of the relative nitrite levels in the media of piglet PA endothelial cells transfected with ASL siRNA vs. NS siRNA in response to L-Arginine (10 mM) treatment (for 60 minutes) with and without L-NMMA (500 μM) or D-Arginine as control (*p< 0.01, **p<0.005). The experiment was performed in triplicates. NS: Non-significant.

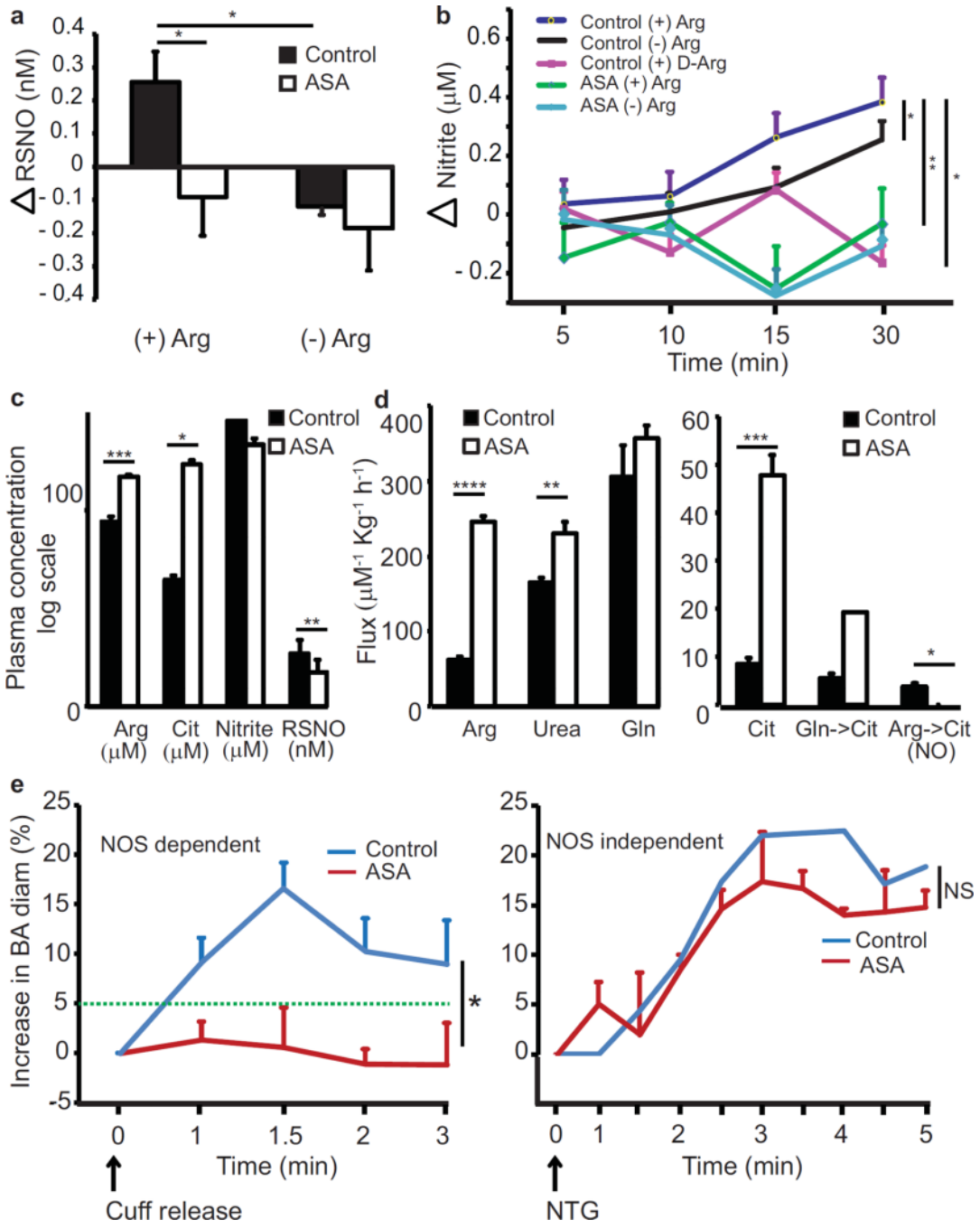

Figure 5. ASL deficient human cells and patients do not efficiently generate NO from arginine

(**a**) RSNO production (ΔRSNO) in control vs. ASA fibroblasts supplemented with L-arginine (1 mM) for 24 hours (*p<0.05). (**b**) Nitrite production (ΔNitrite) in control vs. ASA fibroblasts in response to listed treatments over 30 minutes (ANOVA, *p<0.05 **p<0.0005). (**c**) Human ASA subjects (n=3) plasma arginine, citrulline, nitrite and RSNO levels as compared to controls. (*p<0.05 **p<0.005 ***p<0.0005). (**d**) Dynamic metabolite flux measurements in ASA vs. control subjects. **Left panel**: Arginine and urea flux in ASA subjects (n=3) vs. controls (n=3). **Right panel**: Citrulline flux, fractional transfer of the amido-nitrogen from ^{15}N-glutamine to ^{15}N-citrulline, and fractional transfer of guanidine

nitrogen from $^{15}N_2$-arginine to ^{15}N-citrulline (a marker of NO production) in ASA subjects compared to controls, (GLN-Glutamine; CIT- Citrulline, ARG- Arginine) (*p<0.05 **p<0.005 ***p<0.0005 **** p<0.00005). (**e**) Brachial artery (BA) mean percentage vasodilatation measured using Doppler ultrasound. **Left Panel:** NOS dependent flow mediated relaxation. The green dashed line represents the lower normal limit for vessel dilation (*p<0.05). **Right Panel:** NOS independent relaxation stimulated by 0.4 mg of sublingual nitroglycerin. ASA subjects (n=2), controls (n=3). BA - Brachial Artery; NTG - nitroglycerin.

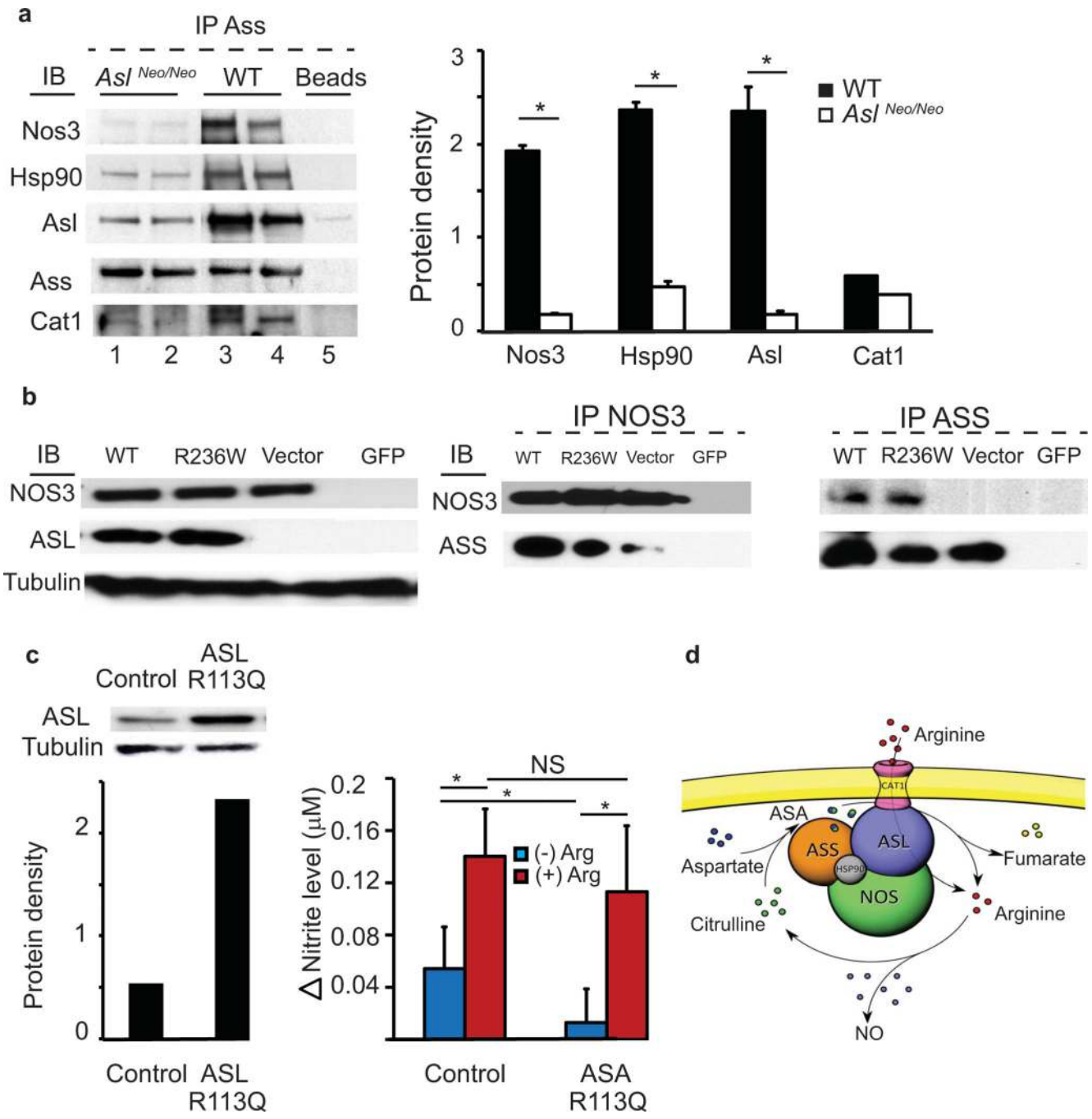

Figure 6. ASL is required to maintain an NO synthetic complex

(**a**) Immunoprecipitation with antibody to ASS and subsequent immunoblots (IB) with listed antibodies of lung protein lysates from *Asl*$^{Neo/Neo}$ (lanes 1–2) and WT mice (lanes 3–4) (n=2 each). On the right is the densitometric quantification from 3 separate studies performed from a total of 9 WT and 12 *Asl*$^{Neo/Neo}$ mice (*p<0.05). (**b,c**) **Effects of ASL catalytic site mutations on complex formation and NO production.** (**b**) Immunoprecipitation with ASS and NOS3 antibodies of COS7 cells transfected with either WT ASL or R236W ASL, empty plasmid or with GFP and NOS3. **Left panel**: A western analysis showing expression of the ASL and NOS3. **Right panel**: Immunoprecipitation with

antibody to NOS3 or to ASS followed by immunoblot as listed. The blots are representative of three different experiments. **(c)** Nitrite production in response to arginine in ASA cells expressing catalytically inactive ASL (*p<0.05). **Left panel**: Western blot showing the expression of ASL in ASA null cells transduced with ASL R113W vs. control fibroblasts. **Right panel**: Nitrite levels measured at 15 minutes with and without addition of arginine to the medium (*p<0.05). (**d**) Model for the NOS complex in arginine channeling. Arginine from *de novo* cellular synthesis by ASL or via transport from extracellular pools by CAT-1 can be channeled to NOS via ASL.