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Atrial natriuretic peptide is negatively regulated by microRNA-425

Pankaj Arora,^{1,2,3,4} Connie Wu,⁵ Abigail May Khan,⁶ Donald B. Bloch,^{7,8} Brandi N. Davis-Dusenbery,⁹ Anahita Ghorbani,^{1,2} Ester Spagnoli,⁵ Andrew Martinez,^{1,3} Alicia Ryan,^{1,3} Laurel T. Tainsh,⁵ Samuel Kim,³ Jian Rong,^{10,11} Tianxiao Huan,^{10,11} Jane E. Freedman,¹² Daniel Levy,^{10,11} Karen K. Miller,¹³ Akiko Hata,¹⁴ Federica del Monte,¹⁵ Sara Vandenwijngaert,¹⁶ Melissa Swinnen,¹⁶ Stefan Janssens,¹⁶ Tara M. Holmes,¹⁷ Emmanuel S. Buys,⁵ Kenneth D. Bloch,^{1,2,5} Christopher Newton-Cheh,^{1,2,3,4} and Thomas J. Wang^{1,2,18}

¹Cardiology Division, ²Cardiovascular Research Center, and ³Center for Human Genetic Research, Massachusetts General Hospital, Boston, Massachusetts, USA. ⁴Program in Medical and Population Genetics, Broad Institute, Cambridge, Massachusetts, USA.

⁵Department of Anesthesia, Critical Care, and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts, USA.

⁶Division of Cardiovascular Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA.

⁷Division of Rheumatology, Allergy, and Clinical Immunology and ⁸Center for Immunology and Inflammatory Diseases,

Massachusetts General Hospital, Boston, Massachusetts, USA. ⁹Stem Cell and Regenerative Biology, Harvard University, Cambridge, Massachusetts, USA. ¹⁰Framingham Heart Study of the National Heart, Lung, and Blood Institute and Boston University School of Medicine, Framingham, Massachusetts, USA. ¹¹Center for Population Studies and Division of Intramural Research, National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland, USA. ¹²Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA. ¹³Division of Endocrinology,

Neuroendocrine Unit, Massachusetts General Hospital, Boston, Massachusetts, USA. ¹⁴Cardiovascular Research Institute, UCSF, San Francisco, California, USA. ¹⁵Cardiovascular Institute, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA. ¹⁶Department of Cardiovascular Sciences,

Gasthuisberg University Hospital, University of Leuven, Leuven, Belgium. ¹⁷Clinical Research Center, Massachusetts General Hospital, Boston,

Massachusetts, USA. ¹⁸Division of Cardiovascular Medicine, Vanderbilt University, Nashville, Tennessee, USA.

Numerous common genetic variants have been linked to blood pressure, but no underlying mechanism has been elucidated. Population studies have revealed that the variant rs5068 (A/G) in the 3' untranslated region of *NPPA*, the gene encoding atrial natriuretic peptide (ANP), is associated with blood pressure. We selected individuals on the basis of rs5068 genotype (AG vs. AA) and fed them a low- or high-salt diet for 1 week, after which they were challenged with an intravenous saline infusion. On both diets, before and after saline administration, ANP levels were up to 50% higher in AG individuals than in AA individuals, a difference comparable to the changes induced by high-salt diet or saline infusion. In contrast, B-type natriuretic peptide levels did not differ by rs5068 genotype. We identified a microRNA, miR-425, that is expressed in human atria and ventricles and is predicted to bind the sequence spanning rs5068 for the A, but not the G, allele. miR-425 silenced *NPPA* mRNA in an allele-specific manner, with the G allele conferring resistance to miR-425. This study identifies miR-425 as a regulator of ANP production, raising the possibility that miR-425 antagonists could be used to treat disorders of salt overload, including hypertension and heart failure.

Introduction

The average adult from the US consumes nearly 10 grams of salt per day, far in excess of the recommended amounts (1, 2). High-salt intake is linked to hypertension and cardiovascular disease (3, 4). The natriuretic peptide system plays a central role in the physiologic response to salt intake. Synthesized by the heart in response to increased intravascular volume; atrial and B-type natriuretic peptides (ANP and BNP, respectively) bind their receptors, particulate guanylyl cyclases, which catalyze and promote the production of cyclic guanosine monophosphate (cGMP); and promote natriuresis, diuresis, and vasodilation.

Evidence from population genetic studies suggests that variation in the plasma levels of natriuretic peptides may alter susceptibility to cardiovascular disease via effects on blood pressure. Common SNPs in the chromosomal region containing *NPPA* and *NPPB*,

the genes encoding the ANP and BNP propeptides, are associated with circulating levels of the natriuretic peptides (5). Large-scale epidemiologic studies have established that the same variants are associated with blood pressure (5, 6). The SNP that is associated with plasma ANP levels most strongly is rs5068 (A/G): carriers of the rs5068 minor G allele have a 15% lower risk of hypertension than do those with 2 copies of the major A allele.

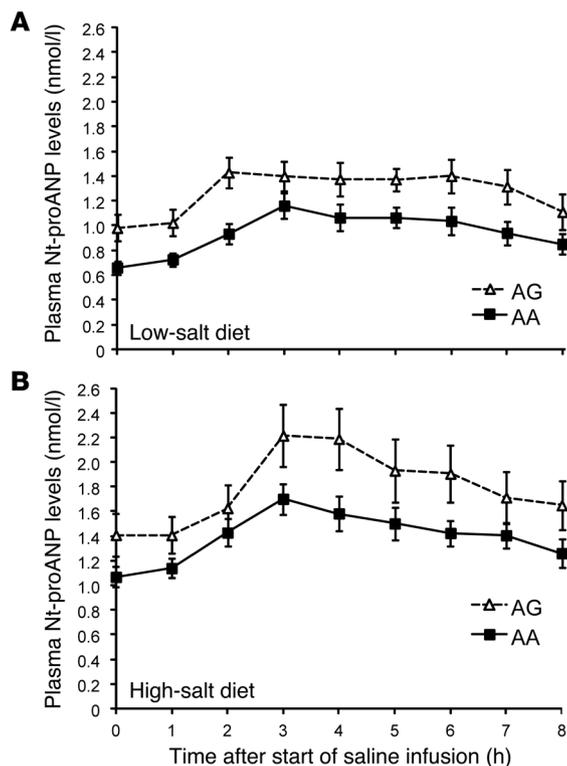
As with all complex traits, the majority of blood pressure-related SNPs are noncoding, and they are typically located within large blocks of correlated SNPs by virtue of linkage disequilibrium. Thus, identifying causal SNPs and the mechanisms by which they act presents significant challenges. Indeed, although genome-wide association studies have yielded a long list of candidate SNPs associated with blood pressure, the only variants for which a mechanism has been established are rare mutations in families with Mendelian forms of hypertension or hypotension (7).

The rs5068 SNP is located in the 3' untranslated region (UTR) of the *NPPA* gene. It has recently been recognized that small noncoding RNAs called microRNAs (miRNAs) can play a role in posttranscriptional regulation of gene expression by binding to 3'UTRs (8, 9). Binding of miRNAs to mRNAs typically requires complementarity of a seed sequence at positions 2–7

Authorship note: Kenneth D. Bloch, Christopher Newton-Cheh, and Thomas J. Wang contributed equally to this work and are co-senior authors.

Conflict of interest: Pankaj Arora, Kenneth D. Bloch, Christopher Newton-Cheh, and Thomas J. Wang are named as coinventors on a patent application relating to the use of miRNAs for the treatment of hypertension and other disorders.

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**Figure 1**

The effect of genotype and dietary sodium on plasma Nt-proANP levels during saline challenge is shown after 1 week of a (A) low-salt diet or (B) high-salt diet ($P = 0.018$ for genotype effect, $P < 0.001$ for diet and saline effects). Samples for measurement of plasma Nt-proANP levels were obtained at baseline and hourly for 8 hours after the start of saline infusion. AA, rs5068 major homozygote; AG, rs5068 heterozygote.

genotype effect). The transition from a low- to high-sodium diet was associated with a 55% increase in Nt-proANP levels ($P < 0.001$), a difference that was similar in both genotype groups (diet-by-genotype interaction, $P > 0.8$). AG individuals on a low-sodium diet had plasma Nt-proANP concentrations that were comparable to those of AA individuals on a high-sodium diet. These findings suggest that rs5068 influences the “set point” of circulating ANP on both low- and high-sodium backgrounds. The magnitude of the genetic effect is similar to that of a marked (20-fold) change in salt intake.

To ascertain whether rs5068 influences the ability of ANP levels to increase in response to intravascular volume expansion, plasma Nt-proANP levels were measured during and after a saline challenge. In the group as a whole, saline administration increased plasma Nt-proANP levels by 64% on a low-sodium diet background (Figure 1A) and 59% on a high-sodium diet background (Figure 1B). Mean plasma Nt-proANP concentrations at all time points during and after the saline infusion were higher in AG individuals than in AA individuals ($P = 0.018$ for genotype effect). There was no evidence of a saline-by-genotype interaction ($P = 0.84$).

After 1 week on a low-sodium diet, saline administration increased plasma cGMP levels in both AG and AA individuals (Supplemental Figure 1A). After 1 week on a high-sodium diet, saline administration increased plasma cGMP levels by 37% in AG individuals and 16% in AA individuals ($P = 0.03$ for genotype effect and $P < 0.001$ for saline infusion; Supplemental Figure 1B). The associations of genotype and cGMP levels were consistent with those observed for circulating ANP levels.

Among the normotensive, young adults in our genotype-directed study, no significant increases were noted in systolic or diastolic blood pressure with the transition from a low-sodium diet to a high-sodium diet, before or after saline infusion. However, there were trends toward lower systolic blood pressures in AG individuals compared with those in AA individuals after the high-salt diet (112 vs. 117 mmHg; $P = 0.10$) and after the saline infusion on a low-sodium background (109 vs. 114 mmHg; $P = 0.06$; Supplemental Table 2). We did not observe an effect of rs5068 genotype on urine volume or sodium excretion in response to saline infusion ($P > 0.3$).

Plasma N-terminal proBNP (Nt-proBNP) and mature BNP levels were higher on a high- than on a low-sodium diet and after saline infusion, but levels did not differ by rs5068 genotype (Supplemental Figure 2, A and B, and Supplemental Figure 3, A and B; $P > 0.5$). These findings support the specificity of the effect of the rs5068 variant on ANP levels.

Although cardiac tissues are primarily responsible for ANP biosynthesis, the *NPPA* gene is expressed at low levels in a wide variety of cell types. We examined whether *NPPA* mRNA levels in the blood of 2,246 Framingham Heart Study participants varied by rs5068 genotype. *NPPA* expression was higher in individuals with at least 1 copy of the G allele ($n = 203$) than in AA

of the 5' end of the miRNA (10). We hypothesized that rs5068 is a causal variant and that the mechanism of its effect on ANP levels involves interference with miRNA binding.

Results and Discussion

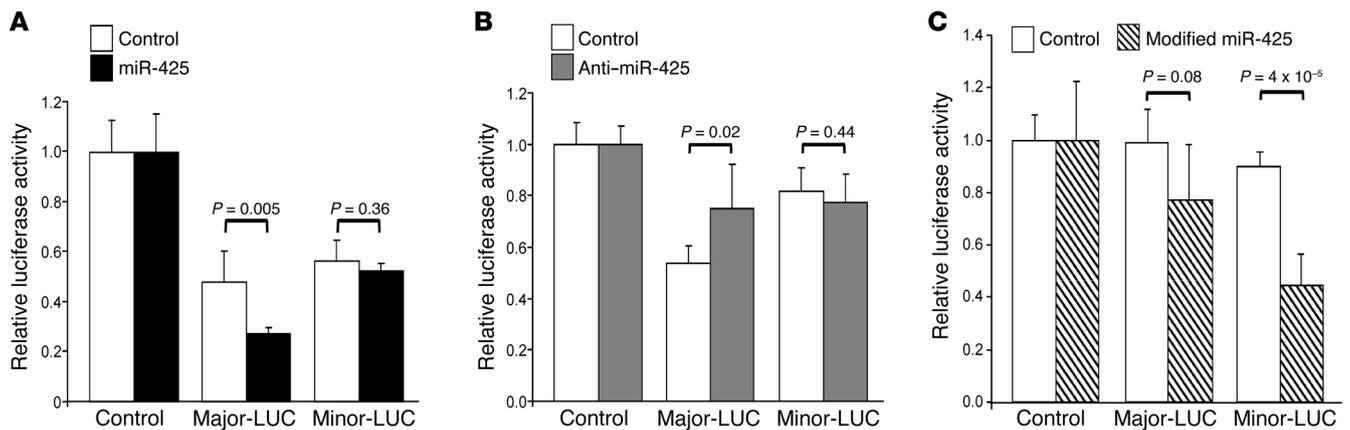
Epidemiologic studies have shown differences in plasma natriuretic peptide concentrations among rs5068 genotypes (5), but these differences were identified in the context of random salt intake and other sources of variation inherent to community-based studies. To minimize this variation and provide mechanistic insights into the association of rs5068 with ANP levels and blood pressure, we undertook a high-resolution physiologic study of healthy subjects who were selected on the basis of their rs5068 genotype and maintained on defined low- and high-salt diets.

We genotyped the rs5068 variant in 699 healthy, normotensive individuals of European ancestry between the ages of 18 and 40. Overall, 645 (92%) individuals had 2 copies of the major A allele (AA), and 54 (8%) had at least one copy of the minor G allele (AG or GG). Detailed characteristics of the 23 AA individuals and 8 AG individuals who participated in the physiologic study are shown in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI67383DS1).

Subjects were placed on a study diet for 2 weeks, consisting of 1 week on a high-sodium diet (200 mEq/d) and 1 week on a low-sodium diet (10 mEq/d), in random order. Mean 24-hour urine sodium was 22 mmol after a week on the low-sodium diet and 142 mmol after a week on the high-sodium diet. AG individuals had higher plasma levels of N-terminal proANP (Nt-proANP) than AA individuals after 1 week on either a low- or a high-sodium diet (49% and 32%, respectively; $P = 0.016$ for overall



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**Figure 2**

Allele-specific effects of miR-425, anti-miR-425, and a modified miR-425 on luciferase activity encoded by firefly luciferase-*NPPA* 3'UTR constructs. The ratio of firefly luciferase activity to renilla luciferase activity was normalized to that in cells transfected with a plasmid directing expression of luciferase without the *NPPA* 3'UTR (relative luciferase activity). Data are expressed as mean \pm SEM ($n = 6$). Each experiment was repeated 3 times with similar results. **(A)** miR-425 reduces activity of the *NPPA* major-LUC construct but not the *NPPA* minor-LUC construct. **(B)** Anti-miR-425 increases activity of the *NPPA* major-LUC construct but not the *NPPA* minor-LUC construct. **(C)** A modified miR-425 targeting the *NPPA* 3'UTR minor allele reduces activity of the *NPPA* minor-LUC construct but not the *NPPA* major-LUC construct.

($n = 2,043$) individuals ($P = 2 \times 10^{-18}$). We also measured *NPPA* mRNA levels in lymphoblasts (Coriell Institute) with AG ($n = 4$) and AA ($n = 5$) genotypes. *NPPA* mRNA levels were 2-fold higher in the cells with the AG genotype ($P = 0.04$). These findings suggest that rs5068 acts by altering *NPPA* gene transcription or mRNA stability, rather than by altering secretory mechanisms or the clearance of the peptide from the circulation.

To identify an inhibitory miRNA that targets the *NPPA* rs5068 allele associated with lower plasma ANP levels, miRNA databases (miRanda, ref. 11; TargetScan, ref. 10; and MicroSNiPer, ref. 12) were screened to generate a list of miRNAs predicted to interact with the *NPPA* 3'UTR containing the rs5068 A allele. We identified 3 miRNAs that were predicted to bind at least 7 bases of the *NPPA* mRNA spanning the rs5068 A allele: miR-425, miR-4770, and miR-196a-3p (Supplemental Figure 4). miR-425 and miR-4770, but not miR-196a-3p, were detected in cardiac tissues from heart transplant donors without cardiovascular disease ($n = 5$). miR-425 levels were similar in left atrium and left ventricle. In a separate study, we found that miR-425 levels in left atrial ($n = 3$) and ventricular tissues ($n = 4$) obtained from patients undergoing aortic valve replacement for aortic stenosis were similar to those in left ventricular tissues from heart transplant donors ($n = 3$).

miRNA function was assessed in COS-7 cells transfected with luciferase reporter constructs containing the 3'UTR from *NPPA* major or minor alleles (major-LUC and minor-LUC, respectively).

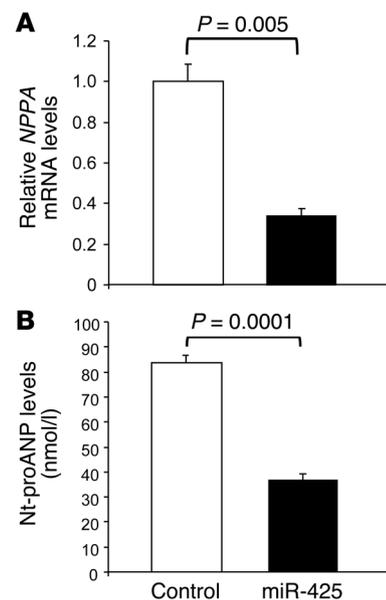
Figure 3

miR-425 transfection reduces *NPPA* mRNA levels and secretion of Nt-proANP immunoreactivity in human cardiomyocytes derived from induced pluripotent stem cells. Cardiomyocytes ($\sim 4 \times 10^5$ per well) were transfected with miR-425 or a control miRNA. Twenty-four hours later, cells were washed and incubated in 1 ml of media. After an additional 48 hours, cells and media were harvested. **(A)** *NPPA* mRNA levels relative to those in cells transfected with control miRNA. **(B)** Concentrations of Nt-proANP immunoreactivity in the cell supernatant. Data are expressed as mean \pm SEM ($n = 4$).

Transfection of miR-425 reduced relative luciferase activity in cells containing the major-LUC construct ($P = 0.005$; Figure 2A) but not in cells containing the minor-LUC construct. Transfection of miR-4770 and miR-196a-3p failed to alter relative luciferase activity ($P = \text{NS}$; data not shown).

Having determined that miR-425 transfection decreased luciferase activity in the presence of the A but not the G allele, we tested whether endogenous miR-425 levels regulate *NPPA* expression. Transfection of an anti-miR-425 increased relative luciferase activity in cells containing the major-LUC construct ($P = 0.02$; Figure 2B) but not in cells containing the minor-LUC construct.

To confirm that the single base pair change at rs5068 was sufficient to alter the binding of miR-425 to the *NPPA* 3'UTR, we constructed a modified miR-425 in which a single base in the





seed sequence was changed to complement the minor G allele of rs5068. In contrast to miR-425, the modified miR-425 suppressed expression of the minor-LUC construct ($P = 4 \times 10^{-5}$; Figure 2C) but not the major-LUC construct.

We examined whether the *NPPA* allele-specific effects of miR-425 lead to altered production of the ANP peptide. In COS-7 cells transfected with an *NPPA* cDNA expression plasmid containing the rs5068 major A allele, cotransfection of miR-425 reduced release of Nt-proANP into the media ($P = 0.008$, Supplemental Figure 5). In contrast, transfection of miR-425 did not alter Nt-proANP release from cells cotransfected with the *NPPA* expression plasmid containing the rs5068 minor G allele ($P = 0.40$; $P = 1 \times 10^{-5}$ for genotype effect).

To determine whether miR-425 could regulate endogenous *NPPA* gene expression, we transfected the miRNA into human cardiomyocytes derived from induced pluripotent stem cells. Compared with transfection of a negative control miRNA, transfection of miR-425 into cardiomyocytes reduced *NPPA* mRNA levels by 66% ($P = 0.0005$; Figure 3A) and secretion of Nt-proANP by 56% ($P = 0.00001$; Figure 3B).

Taken together, these findings provide strong evidence that rs5068 is the causal variant underlying the previously reported blood pressure association, clarify the molecular mechanism by which rs5068 acts on ANP levels, and identify miR-425 as a novel regulator of the natriuretic peptide system.

Our findings illustrate the potential importance of interactions among miRNAs, polymorphisms in noncoding regions of the genome, and responses to environmental factors such as salt intake. It has been postulated that interference with miRNA binding is an important mechanism by which noncoding genetic variants identified by genome-wide association studies may exert their actions. However, to our knowledge, this study provides the first example of this phenomenon for a validated blood pressure association. Interestingly, rs5068 had a substantial effect on the ANP “set point,” without altering the responsiveness of ANP to salt loading. This observation could have implications for future therapies based on miR-425, as it might be desirable to shift basal plasma ANP levels without changing the ability of the system to respond to stimuli.

Several limitations of the study merit consideration. We studied young, healthy, normotensive volunteers in order to minimize interference from comorbidities or medication use. In individuals with hypertension or older age, the transition from low- to high-sodium diet has been associated with an increase in blood pressure (3). Our study was not designed to examine whether the rs5068 variant is associated with salt-induced changes in blood pressure, because 1 week of a high-salt diet does not raise blood pressure in healthy, normotensive individuals (13) and because the association of rs5068 with blood pressure and hypertension has already been established (5, 6). In addition, we studied only individuals of European ancestry, because, to date, it has not been established that rs5068 is associated with plasma ANP levels in populations of non-European ancestry, in whom the minor allele frequency is exceedingly low or zero.

In summary, we have identified a novel mechanism underlying blood pressure regulation and salt homeostasis by way of a common single base pair change in the *NPPA* gene that prevents bind-

ing of miR-425 and results in higher ANP levels. Our findings illustrate that common genetic variants can have a relatively large influence on physiologic responses, even if their association with clinical traits is more modest, presumably due to the multifactorial determination of these traits and compensatory mechanisms. Indeed, the genetic effect of rs5068 on circulating Nt-proANP levels is comparable to the environmental change induced by switching from an extremely low-salt diet (230 mg/d) to a diet with salt content typical of a Western diet (4,600 mg/d). Future studies are warranted to determine whether knowledge of rs5068 genotype could facilitate tailored strategies for the prevention and treatment of hypertension. Our findings also raise the possibility that antagonists of miR-425-mediated suppression of *NPPA* expression could be used to treat disorders of salt overload, including hypertension and its complications.

Methods

A detailed description of the methods is provided in the Supplemental Methods.

Statistics. Repeated-measures ANOVA was used to assess the effect of genotype, diet, and intravenous saline on plasma Nt-proANP, Nt-proBNP, BNP, and cGMP levels. Gene expression data from the Framingham Heart Study have been deposited in dbGAP (National Center for Biotechnology Information, Bethesda, Maryland, USA; accession no. phs000007.v19.p7). For relative luciferase activity, mRNA levels, and culture medium Nt-proANP concentrations, we performed 2-sample or paired *t* tests, as appropriate. All analyses were conducted using SAS. A 2-sided *P* value of less than 0.05 was considered statistically significant. Data are presented as mean \pm SEM in the figures.

Study approval. The protocol was approved by the Partners Human Research Committee. All subjects provided informed consent.

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Address correspondence to: Kenneth D. Bloch, Anesthesia Center for Critical Care Research, Department of Anesthesia and Critical Care, Jackson 4, 55 Fruit Street, Boston, Massachusetts 02114, USA. Phone: 617.724.9540; Fax: 617.724.7768; E-mail: kdbloch@partners.org. Or to: Christopher Newton-Cheh, Center for Human Genetic Research, Massachusetts General Hospital, 185 Cambridge St. CPZN 5.242, Boston, Massachusetts 02114, USA. Phone: 617.643.3615; Fax: 617.249.0127; E-mail: cnewtoncheh@chgr.mgh.harvard.edu. Or to: Thomas J. Wang, Division of Cardiovascular Medicine, Vanderbilt University, 2220 Pierce Avenue, 383 PRB, Nashville, Tennessee 37232, USA. Phone: 615.936.1717; Fax: 615.936.2029; E-mail: thomas.j.wang@vanderbilt.edu.

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