Koch's Postulates and the Digitalis-Like Factor

Norman K. Hollenberg, and Steven W. Graves

Substantial, but still circumstantial evidence, supports strongly a role for a circulating digitalis-like factor in the pathogenesis of salt-sensitive hypertension. Although supported by many lines of evidence, this intriguing concept remains controversial, in large part because the responsible factor has proven to be very elusive. A very large number of candidates from a wide range of chemical classes have been proposed. Indeed, the large number of candidates, none supported by absolutely definitive evidence, has contributed to the controversy. In this essay, we have attempted to define the information that will be required before a candidate becomes widely accepted. Because the current situation resembles so strikingly the situation late in the nineteenth century – when efforts focused on the attempt to identify a specific micro-organism as the agent responsible for specific disease – we employed Koch's postulates as the organizing principle. The challenge faced by Robert Koch over a century ago is identical to the challenge that we face today. (*Hypertens Res* 1995; 18: 1-6)

Key Words: Koch's postulates, digitalis-like factor, sodium hypertension, Na,K-ATPase

Our decision to open our essay on the digitalis-like factor with a review of medical events in the mid-to-late 19th century reflects a firm belief that it is worthwhile to remind ourselves, every so often, that one either learns from history or repeats it. The lessons learned well before the 19th century closed have an important message for us as the 20th century comes to an end.

In a chapter with the intriguing title "The Germ Theory Before Germs", Nuland (1) pointed out that one of the problems faced by Ignac Semmelweis involved unfortunate timing. His ingenuity uncovered the importance of hand washing nine years before Pasteur demonstrated that bacteria caused putrefaction. Indeed, it was to be another twenty years until Lister demonstrated in the 1860's that wound infections, since they are caused by bacteria, can be transmitted by doctors' hands (1-3). In a remarkable shift of "informed opinion", bacteria during the next decade moved from a stage in which they could explain nothing to a stage in which they could explain everything. They are ubiquitous.

The genius of Robert Koch created order from the chaos with his publication of the "Etiology of traumatic infective diseases" in 1879 (2). Although his paper dealt with wound infections, as had that of Lister, he considered the more general problem of connecting specific bacteria with specific effects. The issue involved whether the bacteria were a consequence of the infection, or its cause, or an innocent bystander. To address these issues, Koch de-

veloped a series of principles which were expanded and refined to form what came to be known as "Koch's Postulates" (2). As obvious as they are today, they were revolutionary in their time – and as pointed out above – too often have been forgotten.

Koch identified four logical requirements. The first was that the organism must be present in every case of the disease. The second was that the organism could be identified in pure culture. The third involved transfer of the organism to another animal, which reproduced the disease. Fourth, the organism could then recovered from the inoculated animal and again grown in pure culture. Koch's application of these criteria to the anthrax bacillus led Julius Cohnheim, one of the giants of 19th century medicine to conclude: "it leaves nothing more to be proved. I regarded it as the greatest discovery ever made with bacteria" (1).

These rules of logic are obviously applicable broadly and have thereby influenced much of the evolution of medicine (1-3). With appropriate modification, they have been as crucial in the research on hormones. As pointed out by Haber (4), the elements involving transfer of the vector in Koch's postulates became the ablation-replacement experiment in endocrinology. In this experiment, the investigator first documents the physiological implications of absence of the hormone, and then the response to hormone replacement (4). When that experiment became impossible, pharmacological interruption of the system or immunoneutralization pro-

From the Brigham and Women's Hospital and Harvard Medical School, Departments of Medicine and Radiology, 75 Francis Street, Boston, U.S.A.

Since our manuscript was submitted, another review of this subject with substantial overlap in the conclusion with ours has appeared: Doris PA: Regulation of Na, K-ATPase by endogenous ouabain-like materials. *PSEBM* 1994; 205:202-212.

Address for Reprints: Norman K. Hollenberg, M.D., Ph.D., Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, U.S.A.

Received September 7, 1994.

Table 1 Candidates as a Circulating Digitalis-Like Sodium Pump Inhibitor

I. Lipids	
(i)	Fatty acids
(ii)	Lysophosphatidyl choline
(iii)	HETE pathway
II. Steroids	
(i)	DHEA-S
(ii)	Progesterone
(iii)	Ouabain
(iv)	Digoxin
(v)	Bufalin, 19-norbufalin
(vi)	Bile salts
III. Peptides	
(i)	MSH
(ii)	Endotoxin

Table 2 Pitfalls

(1)	Inadequate assay specificity and range
(2)	Ignoring potency of known candidates
(3)	Problems reflecting the source of the material
(4)	Assumption of stability
(5)	Inadequate purification

vided an analogous pivotal element. In the case of endogenous hormonal systems, an additional logical element involved documenting the endogenous biosynthesis – location and enzymatic pathway. When these criteria are met, one is left – like Julius Cohnheim – with no doubt concerning the role of a hormone in the body economy.

Our goal in this essay is to develop this theme and apply it to the problem of the digitalis-like factor – a putative hormone which many consider to mediate normal sodium homeostasis and volume control, and contribute to the pathogenesis of sodium – sensitive hypertension. Despite a prodigious literature, the subject remains highly controversial – in large part, we believe, because the principles so carefully delineated by Koch have often not been applied.

The Candidates

Because the rules of logic have not been adequately applied, there has been not only an extraordinary number of individual chemical species suggested as the candidate, there has been an extraordinary number of chemical classes: Table 1 enumerates several of the proposed chemical classes, with a few examples of each, and is not intended to be exhaustive. The primary references for each candidate can be found in a series of recent review articles (5-12).

The Pitfalls

In general, this diversity of candidates reflects the

Fig. 1. Relation between sodium pump inhibitor concentration and inhibition. Note the typical mass action kinetics shown by ouabain, and the precipitous inhibition shown by free fatty acids (FFA) and by dehydroepiandrosterone sulphate (DHEA-S).

fact that often single assays of limited specificity were employed in monitoring DLF purification. Typically, this has been either a chemical assay of sodium, potassium ATPase activity or a radioimmunoassay for digoxin. Rarely were other criteria examined. This and other pitfalls that have complicated this field are summarized in Table 2. The chemical assay of sodium, potassium ATPase activity is sensitive to an array of interfering specific and non-specific influences. For example, claims that free fatty acids, lysophosphatidyl choline and dehydroepiandrosterone (DHEAS) sulfate should be considered candidates depended on their activity in a biochemical assay. Examination of Fig.1 compares inhibition of sodium potassium ATPase induced by ouabain, and by a number of proposed candidates. The information used to construct the figure was taken from the relevant publications (13, 14). Note that ouabain, the standard, is relatively inactive compared to most hormones – with a K_i well above 10^{-7} M – and that the relation between ouabain concentration and inhibition followed a typical sigmoid, mass action relationship, developing over several orders of concentration. The other candidates, on the other hand, are even less potent and show a precipitous onset and steep range of activity, a concentration effect suggestive of a non-specific, physical action. In the case of the lipids, it may represent a “detergent” effect.

Radioimmunoassay does not measure bioactivity. It examines only a portion of the molecule, and has been shown to be misleading when employed alone (15). Indeed, the fact that antisera directed against digoxin vary widely in their sensitivity to the endogenous crossreactivity agent appears not to have been considered in many research strategies.

Many investigators consider it crucial that a relevant bioassay system be employed as part of the assay strategy. Unfortunately, bioassay systems are

complex and often poorly understood. For example, if one assumes that the candidate acts as the natriuretic agent, and makes natriuresis a sole or crucial criterion, the fact that blockade of sodium reabsorption in one portion of the nephron can be offset by increased reabsorption elsewhere can limit the information obtained from this bioassay. In the case of vascular smooth muscle, it has been recognized only recently that the contractile response resulting from sodium pump inhibition differs dramatically from the contractile response resulting from other known agonists (16). In the case of sodium pump inhibition there is a striking delay in the contractile response, often measured in hours. Virtually, every research strategy, involving vascular smooth muscle contraction employed to study DLF then, would have missed the relevant contractile response.

The decision concerning the source of the material can be crucial. In some cases, the investigators have ignored the physiological role of the putative agent, and have made no attempt to study affected populations or, more importantly, to employ volume expansion as a stimulus before the samples are collected. Indeed, a comparison of samples collected for volume-expanded and volume-contracted individuals can serve as a useful criterion for candidacy.

One strategy, that given attention above, involves the attempt to isolate and purify a candidate from a biological source. A second approach, and one with substantial validity, involves assessing defined pure biochemicals as candidates. In many cases, especially the endogenous steroids with an already known alternative hormonal function, some weak DLF activity has been found in one or more assay systems. Too often investigators have ignored the fact that the high concentrations required for "DLF" activity in the assay systems are never achieved *in vivo*.

Unstated Assumptions

Every research strategy reported has made a fundamental assumption, although it is rarely stated. In each case, the approach has assumed that the putative hormone is sufficiently stable that techniques required to stabilize labile agents have not been employed. Prolonged intervals between collection, purification steps and in some cases, assay and harsh conditions for removing it, especially from plasma protein, have complicated the process (17). At least one candidate is extremely labile, with a half life measured in hours (18).

There have been many assumptions regarding the appropriateness of the specimen used in DLF isolation. In efforts to isolate DLF, plasma as the source has the advantage that the material obtained must be in the circulation, but the obvious disadvantages include of the limited amount that can be collected from each individual. Moreover, protein binding can be a complicating factor. In one study, a candidate was found to show substantial protein binding, and required harsh, potentially modifying conditions to remove the agent from the protein (19). Urine is

Table 3 Criteria Required to Prove that a Candidate Is an Endogenous Hormone – The Digitalis-Like Factor

(1) Action at physiological concentration
(2) Relevant physiological and clinical correlates
(3) Appropriate biochemical activity
(4) Appropriate bioactivity
(5) Surgical, pharmacologic, or immunological reversal
(6) Biosynthesis

an attractive source because it is freely available and abundant, but many hormones reach urine in only limited quantities because of prior metabolism. Moreover, urine may contain a substantial quantity of factors that interfere in the assay systems (9). Several lines of evidence have suggested that the tissue source could be the hypothalamus (6) or the adrenal (5, 20), and investigators have exploited these observations to attempt to isolate candidates from these tissues. Although this approach might prove to be very productive in the future, the large number of steroids found in the adrenal that appear to have at least weak sodium, potassium ATPase inhibitory activity or crossreactivity in digoxin or ouabain RIA has complicated efforts. In the case of the hypothalamus, the small amount of tissue obtained from each animal, and the time required to collect that material might prove to be crucial for reasons discussed below.

Claims of isolation of a pure agent often have been based on inadequate purification. Application of HPLC has led to a description of "purified to a single peak" or "purified to homogeneity by HPLC", with the unstated assumption that this meant that a single chemical species had been isolated. We have found that application of supercritical fluid chromatography to an HPLC fraction that appeared to be pure, revealed several residual residual peaks, probably reflecting multiple chemical species.

The Criteria : Koch's Postulates Applied to DLF

It is not our intent to be critical of the individual investigators and their efforts, but rather to establish a series of guidelines or criteria to simplify and focus future efforts, reflecting the lessons taught by Robert Koch over one hundred and thirty years ago, and on lessons learned from the review of pitfalls.

In Table 3 are listed a modification of Koch's postulates applied specifically to the issue of the digitalis-like factor – or indeed, any hormone. The first criterion, that the candidate be present in blood or other body fluids in a concentration adequate to induce the biological response of interest is so obvious that one might be tempted to omit it. On the other hand, examination of the literature makes it clear that this criterion has generally been ignored. As one example, the elegant evidence that a material identical to or very similar to ouabain can be found in normal human plasma is beyond

Fig. 2. Relation between change in body weight, as an index of extracellular fluid volume expansion, and serum DLF activity during liberalization of salt and water intake in patients on chronic ambulatory peritoneal dialysis. The increase in digitalis-like activity, reflecting increases in the specific inhibitory molecule only during volume expansion should be a crucial criterion for candidates as the endogenous digitalis-like agent.

debate (21). On the other hand, the concentrations of ouabain reported in the early studies (10) appear to be well below those required to induce a contractile response, for example, in vascular smooth muscle (16). Similarly although progesterone and other progestins have some actions suggestive of an endogenous sodium pump inhibitor (22), the concentration required to act in most assay systems is 100-to-1,000 fold higher than the usual endogenous concentration (23). In contrast, another steroid, dehydroepiandrosterone (DHEA) might achieve levels in cord blood required to act in some assay systems (23).

The second criterion involves relevant clinical or physiological correlations. Specifically, is the concentration of the material elevated in affected individuals and does the concentration vary in appropriate direction and magnitude with the appropriate stimulus? In the case of the digitalis-like factor, the appropriate stimulus would seem to be volume expansion and an increase in total body sodium (5-12). In the many hypertension models examined by Haddy and his coworkers, a volume-dependent factor was a common theme (9). Some investigators have examined the influence of rapid volume expansion with saline (24). Development of an appropriate model is a crucial element in attempts to isolate the hormone. Our group has employed as a useful model the patient with chronic renal failure in whom programmed and predictable shifts in total body sodium can be achieved easily with changes in salt and water intake (25). In this situation, we have been able to show close correlations between changes in body weight as the index of volume expansion, changes in arterial blood pressure, and

changes in the activity of the putative hormone in plasma and in peritoneal dialysate (Fig. 2). Such an observation provides substantial confidence that the material under investigation is, indeed, a logical candidate.

Appropriate activity in biochemical assays is the third criterion. The term "appropriate" includes a requirement that the agent under consideration show affinity for the digitalis-binding site of ATPase that is reversible, that reflects substantial potency, and displays features suggesting an appropriate mass action relationship. Specificity for the binding site for digitalis glycosides includes appropriate sensitivity to the ionic milieu (26). More recently, evidence has accumulated to suggest that like cardiac glycosides, incubation conditions prior to initiating hydrolysis can provide additional information on the mechanism of action for DLF - enzyme interactions (27). Equally important is the requirement that the agents show consistent activity in a range of assay systems. As a minimum, the chemical assay systems should include inhibition of [Na,K]ATPase hydrolysis, the function of the enzyme, and an assessment of binding. The use of radioimmunoassay employing antisera directed against digoxin or other known cardiac glycosides is convenient, but has often led to controversial data. Should this approach be employed, it needs to be in conjunction with at least two other DLF assays - a chemical and a bioassay.

Appropriate bioassay is an absolute requirement, in conjunction with appropriate biochemical assays of activity. Failure to document an appropriate action on vascular smooth muscle, renal sodium handling, or blood pressure at concentrations that can be achieved *in vivo* would have dismissed many candidates at an early stage of evolution. In the case of many digitalis glycosides their cardiac toxicity would have led to death of an assay animal well before a pressor response occurred. On the other hand, the bioassay systems are complex, as indicated above, and also can suffer from a lack of specificity.

The term "anatomic, pharmacologic or immunologic reversal", employed in Table 3, requires explanation. Earlier, we emphasized the point made by Haber that the ablation: replacement experiment is crucial among the lines of evidence proving that a candidate represents an endogenous hormone (4). In the classical experiment, the biological influence of ablation of the source is documented, and reversal of that influence by replacement with the candidate closes the logic loop (4). Early attempts at the experiment have included ablation of portions of the hypothalamus, which led to biological responses consistent with the thesis that an endogenous sodium pump inhibitor in or from this region participates in sodium homeostasis (28).

Unfortunately, both in the case of a hypothalamus and in the case of the adrenal, ablation creates an extraordinarily complex situation for blood pressure control and sodium homeostasis. Haber developed this theme to point out the crucial role played by pharmacological and immunological interruption of the renin - angiotensin system in fulfill-

ing Koch's requirements (4). We suspect that the endogenous digitalis-like factor represents an analogous situation, in which pharmacological or immunological interruption will play a crucial role. Until the endogenous agent is identified, this avenue of investigation cannot be definitive. On the other hand, preliminary efforts have been made with pharmacological probes such as canrenone (29), and antisera directed against digoxin (30).

Finally, given the large number of candidates identified, the ubiquitous distribution of steroids with an action on the sodium pump in many varieties of plants, and growing evidence that these are absorbed and even taken up by tissues such as the adrenal (31), there is an additional criterion that is likely to be required before investigators interested in this field will accept a candidate. We believe that delineation of the biosynthetic pathway is a crucial step in proving that a candidate is an endogenous hormone.

None of the agents currently under consideration as yet satisfies these stringent criteria. We hope that the application of the principles discovered by Koch one hundred and thirty years ago will clarify and simplify what has become a highly contentious and confusing field.

Why Should Interest Continue?

Equally one might ask, given the number of false trails that have been pursued why we, and so many others, have continued to pursue the elusive digitalis-like factor? The answer can be found in the many review articles cited above (5-12). In animal models of volume-dependent hypertension, evidence favoring sodium pump inhibition as at least a contributing factor, is essentially overwhelming. These observations are supported by multiple lines of less direct evidence in humans which is also compatible with a contribution of a circulating sodium pump inhibitor. Indeed, if multiple premature claims announcing the isolation of the digitalis-like factor had not appeared, this would be one of a large number of interesting scientific areas in which identification of a responsible vector was expected momentarily. Disenchantment expressed, we believe, reflects a response to those premature claims. After this article had been completed, a review on the digoxin-like sodium pump inhibitor story appeared from one of the productive groups in this area (32). Many of the sentiments expressed in this editorial echo a statement in that article. It would seem appropriate to close with a quotation from that article... "Now that there is little doubt that endogenous digoxin-like inhibitors of sodium transport exist...", the link between these substances, salt intake vascular tone, must be pursued with increasing vigor." That pursuit, of course, will be easier if the criteria concerning the responsible mediator are employed systematically.

References

- Nuland SB. Doctors: The Biography of Medicine. NY, Alfred A. Knopf, 1988, pp. 238-262.
- McGrew RE: Encyclopedia of Medical History. NY, McGraw Hill Book Co., 1985, pp. 25-30.
- Lyons AS, Petrucelli RJ: Medicine: an Illustrated History. NY, Harry N. Abrams Inc. Publishers, 1987.
- Haber E: The role of renin in normal and pathological cardiovascular homeostasis. *Circulation* 1976; **54**: 849-861.
- Gotto A, Yamada K, Yagi N, Yoshioka M, Sugimoto T: Physiology and pharmacology of endogenous digitalis-like factors. *Pharmacol Rev* 1992; **44**: 377-399.
- Haber E, Haupt GT Jr: The search for a hypothalamic Na^+ , K^+ -ATPase inhibitor. *Hypertension* 1987; **9**: 315-324.
- Graves SW, Williams GH: Endogenous digitalis-like natriuretic factors. *Ann Rev Med* 1987; **38**: 433-444.
- Buckalew VM, Jr, Haddy FJ: Circulating natriuretic factors in hypertension, in Brenner BM, Laragh JH (ed): Hypertension: Pathophysiology, Diagnosis, and Management. Chapter 60, NY, Raven Press, 1990, pp 939-954.
- Wechter WJ, Benaksas EJ: Natriuretic hormones. *Progress Drug Res* 1990; **34**: 232-260.
- Hamlyn JM, Manunta P: Ouabain, digitalis-like factors and hypertension. *J Hypertens* (suppl 7) 1992; **10**: 99-111.
- Graves SW: The sodium pump in hypertension. *Current Opinion Nephrol Hypertens* 1994; **3**: 107-111.
- MacGregor SE, Walker JM: Mini review: inhibitors of the Na^+ , K^+ -ATPase. *Comp Biochem Physiol* 1993; **105C**: 1-9.
- Kelly RA, O'Hara SO, Mitch WE, Smith TW: Identification of NaK:ATPase inhibitors in human plasma as nonesterified fatty acids and lysophospholipids. *J Biol Chem* 1986; **261**: 11704-11711.
- Vasdev S, Longerich L, Johnson E, Brent D, Gault MH: Dehydroepiandrosterone sulfate as a digitalis like factor in plasma or healthy human adults. *Res Comm Chem Pathol Pharmacol* 1985; **49**: 387-399.
- Graves SW, Naomi S, Williams GH, Hollenberg NK: Digitoxin antibody crossreactivity and evaluation of potential candidates for the circulating digitalis-like immunoreactive factor. *Clin Chem* 1994; **40**: 1977-1978.
- Stewart L, Hamilton C, Ingwall J, et al: Vascular smooth muscle response to ouabain. Relation of tissue Na^+ to the contractile response. *Circ Res* 1993; **73**: 1113-1120.
- Hamlyn JM, Ringel R, Schaffer J, et al: A circulating inhibitor of (Na^+K) ATPase associated with essential hypertension. *Nature* 1982; **300**: 650-652.
- Graves SW, Soszynski PA, Tao QF, Williams GH, Hollenberg NK: A labile endogenous Na-pump inhibitor in man. *Biol Chem (Hoppe-Seiler)*. 1994 (in press).
- Graves SW, Sharma K, Chandler AB: Methods for eliminating interferences in digoxin immunoassays caused by digoxin-like factors. *Clin Chem* 1986; **32**: 1506-1509.
- Ludens JH, Clark MA, Robinson FG, DuCharme DW: Rat adrenal cortex is a source of a circulating ouabain-like compound. *Hypertension* 1992; **19**: 721-724.
- Hamlyn JM, Blaustein MP, Bova S, et al: Identification and characterization of a ouabain-like compound from human plasma. *Proc Natl Acad Sci* 1991; **88**: 6259-6263.
- Vinge E, Helgesen-Rosendal S, Backstron T: Progesterone, some progesterone derivatives and urinary

- digoxin-like substances from pregnant women in radioimmuno- and ^{86}Rb -uptake assays of digoxin. *Pharmacol Toxicol* 1988; **63**: 277-280.
23. Burtis CA, Ashwood ER: Tietz Textbook of Clinical Chemistry. Second edition. Philadelphia, PA., W. B. Saunders Co., 1994, pp 2203-2204.
24. Gruber KA, Whitaker JM, Buckalew VMJ: Endogenous digitalis-like substance in plasma of volume expanded dogs. *Nature* 1980; **277**: 743-745.
25. Glatter KA, Graves SW, Hollenberg NK: Sustained volume expansion and [Na,K]ATPase inhibition in chronic renal failure. *Am J Hypertens*, 1994; **7**: 1016-1025.
26. Forbush III B: Cardiotonic steroid binding to Na, K-ATPase. in Hoffman JF, Forbush III B (eds): Structure, Mechanism, and Function of the Na/K Pump. NY, Academic Press, 1983, pp. 167-201.
27. Tao QF Soszynski PA, Hollenberg NK, Graves SW: A sensitive assay for sodium pump inhibition. *Clin Chem* 1994; **40**: 1595-1596.
28. Songu-Mize E, Bealer SL, Caldwell RW: Effect of DOCA-salt treatment duration and anteroventral third ventricular lesions on a plasma-borne sodium pump inhibitor in rats. *J Hypertens* 1987; **5**: 461-467.
29. Manunta P, Tyzack J, Hamilton BP, Hamlyn JM: Augmentation and antagonism of ouabain-induced hypertension. *Hypertension* 1993; **22**: 432 (Abstract).
30. Zidek W, Ottens E, Heckmann U: Transmission of hypertension in rats by cross circulation. *Hypertension* 1989; **14**: 61-65.
31. Kitano S, Morimoto S, Koh E, Ogihara T: The adrenals release accumulated exogenous ouabain at volume expansion in reduced renal mass hypertensive rats. *Hypertension* 1993; **22**: 432 (Abstract).
32. Woolfson RG, Poston L, De Wardener HE: Digoxin-like inhibitors of active sodium transport and blood pressure: the current status. *Kid Int* 1994; **46**: 297-309.